Original Article

SIMPLE DIRECT DRUG SUSCEPTIBILITY TESTS ON SPUTUM SAMPLES FOR EARLY DETECTION OF RESISTANCE IN TUBERCLE BACILLI

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Summary

Background: Direct sensitivity test either by sputum concentrate (DS) or swab method (DSM) set up along with the primary culture would avoid the delay of four or more weeks required for the indirect test. A comparison of these two methods against the standard indirect sensitivity method under routine laboratory conditions is necessary to prove their merit.

Method: Smear positive sputum samples were aliquoted and sensitivity tests were set up by both the direct methods as also an indirect test set up from the primary culture of the same sample.

Results: The agreement with the indirect test results for isoniazid (INH) ranged from 97-98% for the DS method and 93-97% for the DSM method. The corresponding figures were 96-98% by the DS and 94-99% by the DSM method for rifampicin (R). The agreement was less satisfactory for ethambutol (Emb).

Conclusion: This study showed that direct sensitivity tests such as DS and DSM methods can detect most of the cultures resistant to INH and R (MDR) from the time growth appears on the primary culture , even as early as the second week of setting up the tests. **[*Indian J Tuberc 2007; 54:184-189*]**

Key words: *M. tuberculosis*, Direct tests, Measuring resistance

INTRODUCTION

As early as 1969 and 1970, results based on direct sensitivity tests using a concentrated deposit of sputum decontaminated by Petroff's method (DS), as also by sputum swabs, decontaminated using cetrimide (DSM) were published [1,2]. However, these methods had only academic interest during that period, since treatment for TB was not based on drug susceptibility results. Simple and time saving methods are needed today, particularly for previously treated, smear-positive patients whose continued treatment should ideally be based on their drug susceptibility pattern.

Though acceptable definitions emerged for resistance to isoniazid (INH), streptomycin (S) and rifampicin (R) using the two direct sensitivity tests methods, swab direct sensitivity tests for R and ethambutol (Emb) were yet to be standardized [3].

In the present study, direct sensitivity testing for INH, R and Emb by swab (DSM) and concentration (DS) culture methods were compared against the indirect test, which is the gold standard.

MATERIAL AND METHODS

Specimens: Smear positive sputum samples obtained from 151 patients attending the Centre's clinic irrespective of their treatment status formed the study material. To each of the sputum samples, few sterile glass beads were added to homogenize it and the contents were divided into two aliquots of 3-5 ml each for culture by Petroff's method and swab culture method respectively [4, 5].

Smear examination: Sputum smears were examined by fluorescent microscopy and graded as 3+, 2+ or 1+; the least grade consisted of 4 or more but less than 100 bacilli in the entire field examined at high power magnification[6].

Medium: Lowenstein-Jensen (L-J) medium was

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used for culture and drug susceptibility testing [7]. For the standard indirect test, the concentrations of drugs used were 0.2, 1.0 and 5.0 mg/l of INH; 32, 64 and 128 mg/l of rifampicin; and 2, 4 and 8 mg/l of Emb. For both the direct sensitivity tests, 0.2 mg/l of INH; 40 and 64 mg/l of R (R40, R64) and 4 mg/l of Emb (E) were used. In addition, L-J medium containing 500 mg/l of para nitro benzoic acid (PNB) was used with all three drug susceptibility methods as an identification test for *M. tuberculosis.*[8]

Indirect sensitivity tests: Deposit from the aliquots of sputum samples treated by Petroff's method was inoculated onto a pair of L-J media. As and when sufficient growth was observed, an indirect sensitivity test was set up for INH, R and Emb, and also for the control strain *M.tuberculosis* H37RV using the standard method[4,8]. The minimal inhibitory concentration (MIC) was determined at the end of four weeks.

Direct Sensitivity Test (DS): The sputum deposits obtained by Petroff's method were inoculated with a 5 mm loop (27 SWG) onto a pair of plain L-J and drug containing L-J media (INH 0.2; R-40, R-64 and Emb4) and one slope of PNB. The slopes were incubated and examined weekly for 8 weeks and the growth was recorded each week.

Direct Swab Sensitivity Test (DSM): Swab culture was set up from the remaining aliquot of sputum [2]. In brief, 11 sterile moistened swabs were dipped in the sputum and gently rotated and transferred to as many test tubes half filled with 1% sterile cetrimide solution. After one hour, each swab was carefully drained of excess fluid and smeared over the surface of 2 slopes of plain L-J and drug containing L-J Media (INH 0.2; R40, R64 and Emb4) and one slope of PNB. The slopes were incubated and examined weekly for 8 weeks and the level of growth was recorded.

Thus, the results for the direct tests DS and DSM were available from the time growth was first recorded on the plain L-J slopes. For all tests, growth on L-J was graded as 3+ if confluent, as 2+ if there were more than 100 discrete colonies, as 1+ for 20-99 colonies, and the actual count if there were less than 20 colonies. The two direct tests were identified by different series of numbers and read by two independent readers to avoid bias. The concentration culture and indirect tests were read by a third reader who had no access to the results of the direct tests, thereby avoiding giving subjective results.

Interpretation of tests

Indirect tests: Resistance was defined as MIC (based on a 20 colony end point) of 1 mg/l or more for INH, 128 mg/l or more for R, and 8 mg/l or more for Emb.

Direct tests (DS and DSM): The definition of resistance was based on the amount of growth seen on the drug-free medium as already reported.[1,2,3] Thus, when the growth on the drug-free medium was 2+ or more, growth of 1+ or more on the drug-containing medium was defined as resistance to the drug. When growth on the drug-free medium was 1+ or less (i.e. < 100 colonies), any growth on the drug-containing medium was considered to be an indication of resistance to that particular drug. For this purpose, the higher growth observed on the paired slopes was considered for interpretation.

Cultures were classified as sensitive or resistant to the drugs INH, R and Emb by the indirect and direct tests (DS and DSM), depending on their respective definitions.

Analysis: The disagreements, if any, were looked into to determine their statistical significance using the McNemar test and to decide the optimum week for correct interpretation of the results. The two direct tests were compared together as well as with the indirect test to assess their relative merit.

RESULTS

The DS test was set for 118 smear positive samples among which 10 were negative on culture (all 1+ smear grades) and 8 were contaminated on the drug-free L-J medium. Thus, 100 samples remained in the analysis. Of these, 11 were contaminated on the INH medium, one each on the R40 and R64 medium and 9 on the Emb medium.

The comparison with the indirect test was therefore available for 89 tests on INH, 99 on R and 91 on Emb.

Sensitivity tests for INH by DS were available for 64 (72%) samples at 2 weeks, 76 (85%) at 3 weeks and 84 (94%) at 4 weeks. The comparison of the results by DS with that obtained with the indirect test is tabulated based on readings taken at 2, 3 and 4 weeks (Table 1). When INH was analyzed, the extent of agreement was 97% at 2 weeks, 99% at 3 weeks and 98% at 4 weeks. A total of 29 out of 30 resistant samples were detected by DS. The little disagreement there showed no statistical significance to suggest a trend in any one direction. Thus, results available by DS were highly reliable as early as the second week of growth.

Since R40 and R64 gave similar results for R, R64 alone is tabulated, since it is the critical concentration for determining resistance by the indirect test method. The agreement between the two tests was 96% at 2 weeks and 98% at 3 and 4 weeks. In the following weeks, the disagreements were fewer. By week 6, 25 out of 26 resistant strains were detected by DS (not tabulated). At 2 weeks, 3 resistant cultures were read as sensitive but none the other way.

The DS test for Emb showed 94% agreement with the indirect test at 2 weeks, 88% at 3 weeks and 92% at 4 weeks. At 2 weeks, 4 resistant cultures were classified as sensitive. At 3 weeks, the disagreements involved six resistant and three sensitive strains. From week 4, the disagreements were greater both in sensitive and resistant strains.

Direct sensitivity test by the swab method (DSM): Swab cultures and sensitivity tests were set up from all 151 smear-positive samples. Among them, 33 were excluded from the analyses, since they did not have results either by one or both of the methods. Of the remaining sample, 24 showed no result by DSM (17 negative and 7 contaminated), 23 showed no result by DS (10 negative and 13 contaminated) and 14 among them were common to both methods. Among the remaining 118 tests, Emb medium was contaminated for one sample. The comparison of the classification by DSM and the indirect tests are presented in Table 2.

Table 1: Comparison of Direct Concentration Method (DS) with indirect sensitivity test results

Classification based on indirect test	Identical classification obtained with DSM test								
	INH n = 118			R 64 n =118			Emb n= 117		
	2W	3W	4W	2W	3W	4W	2W	3W	4W
Sensitive	47	66	71	53	75	81	58	82	90
Resistant	15	33	37	9	22	28	3	10	14
Per cent Agreement	93	97	96	91	95	97	91	91	94
Results available	67	102	112	68	102	112	67	101	111

Classification based on indirect test	Identical classification obtained with DS test								
	INH n = 89			R 64 n =99			Emb n= 91		
	2W	3W	4W	2W	3W	4W	2W	3W	4W
Sensitive	48	50	55	57	63	70	58	62	71
Resistant	14	25	27	11	19	22	2	6	8
Per cent Agreement	97	99	98	96	98	98	94	88	92
Results available	64	76	84	71	84	94	64	77	86

Table 2: Comparison of Direct Swab Method (DSM) with indirect sensitivity test results

The number of samples with results for INH was 67 (56%), 102 (86%) and 112 (95%) at 2, 3 and 4 weeks respectively. The extent of agreement seen for INH was 93% at 2 weeks, 97% at 3 weeks and 96% at 4 weeks. At 2 weeks, 4 resistant cultures were classified as sensitive by DSM and one sensitive as resistant by DSM. These differences, however, were not statistically significant from the third week onwards, when the disagreements were fewer and a total of 41 out of 43 INH-resistant strains were detected using this method.

The rifampicin test based on R64 showed 91% agreement at 2 weeks, 95% at 3 weeks and 97% at 4 weeks. At 2 and 3 weeks, 5 resistant strains were classified as sensitive by DSM but no sensitive culture appeared to be resistant. This difference was statistically significant ($P<0.02$). In the following weeks the disagreements were fewer and not significant. At sixth weeks, 33 out of 34 R-resistant strains were detected by DSM.

Agreement seen for Emb with the indirect test was 91% at 2 and 3 weeks and 94% at 4 weeks. Misclassification by the DSM method occurred with 6 resistant strains at 2 weeks, 8 at 3 weeks and 6 at 4 weeks, with only one sensitive culture being classified as resistant at 3 and 4 weeks. Again, these differences were statistically significant ($P<0.05$ at 2 and 3 weeks), showing that the DSM method under read resistance in the initial weeks. Results not tabulated showed that the trend was reversed in the later weeks probably as a result of deterioration of the drug in the medium with prolonged incubation. Though 19 out of 23 Emb-resistant strains were detected at 7 weeks by DSM, the method was not extremely reliable at any time during the 8 weeks.

Further analyses were undertaken to compare the two direct tests, DS and DSM, with each other as well as against the indirect test, based on readings at four weeks for all three drugs (Table 3). The number of cultures with results by all three methods was 78 for INH, 85 for R and 77 for Emb. The extent of agreement among the three tests in terms of classifying cultures as sensitive or resistant was as high as 95% for INH, 94% for R and 90% for Emb. One culture which was sensitive to INH was classified as resistant by both the two direct tests, though both were based on 3+ growths on the drug-free medium. One sensitive and one resistant culture to Emb were misclassified by both the direct tests. Rifampicin showed complete agreement by all three methods.

Analyses not tabulated have shown that the mean time for culture positivity at 2-3 weeks was similar when processed by the concentration or by the swab method. However, the grades of positivity were considerably higher with the concentration method; growth of 2+ or more was seen in 87% of cultures by the concentration method compared to 55% by the swab method. The number of cultures with less than 20 colonies was 3 by the concentration method and 22 by the swab method. However, the quantitative difference in growth did not affect the agreement in classification of strains when compared to the indirect sensitivity test.

Table 3: Correlation of identical results obtained by direct tests (DS & DSM) with results of the indirect test

All cultures in the analysis were identified as strains of *M. tuberculosis* based on their susceptibility to PNB and positive Niacin production.

DISCUSSION

Simple, inexpensive speedier drug susceptibility test results in resource poor settings, are urgently needed to control the spread of MDR-TB. In this study, two methods of direct drug susceptibility tests (DS and DSM) yielded 85% of the sensitivity results from the third week. The level of agreement with the indirect sensitivity test was 99% and 97% for INH, 98% and 95% for R and 88% and 91% for Emb by the DS and DSM methods respectively. For INH and R, the agreement was 98.4 and 99.4 respectively. There was no false report of resistance at two weeks for any drug by either method. Analyses of disagreement seen beyond 2 weeks in INH and R were statistically non-significant. With incubation beyond 4 weeks, a few false resistant results occurred with Emb. This could perhaps be attributed to the deterioration of the drug.

Rifampicin susceptibility was assessed based on two concentrations, namely, 40 mg/l, the concentration used internationally in proportion sensitivity method and 64 mg/l the critical concentration differentiating the resistant from the sensitive strains by the MIC method. The two concentrations gave 96% and 97% agreement when compared with the indirect method[12].

Ethambutol gave more discrepant results when compared to the indirect test throughout the 8 weeks of incubation, under reading resistance up to 4 weeks and over reading in excess of 4 weeks, a finding suggestive of the deterioration of the drug in the medium with incubation.

Lack of standardization of the inoculum is the main criticism raised against the reliability of the direct tests. This study showed that the growth was significantly more on the plain medium of the concentration direct test then of the swab direct test, as could be expected. Yet it did not alter the results as the two direct tests were equally accurate in classifying the cultures from the second week for INH and third week for R.

This was possible because the definition for resistance was based on the quantity of growth on the drug containing medium relative to that on the plain medium. This definition evolved in studies in 1969 and 1970, have stood the test of time and can be considered highly reliable.

Unpublished data from this centre has shown that the agreement of indirect sensitivity tests done on the same culture was highly reproducible for INH, R and Emb. The advantage of the swab direct test the concentrate direct test is that it requires no special equipment such as the centrifuge, is less hazardous to the worker since the procedure does not create much aerosol contamination of the area, and requires only one relatively inexpensive chemical reagent, i.e. the commercial cetrimide in 1% solution.

The advantage of the direct tests over the indirect test is that in most samples it gives sensitivity results at the same time as the primary culture. This process not only reduces the turn around time by four weeks, but also contamination by eliminating the step of making a subculture. Most importantly, the results of the direct tests are more closely representative of the bacterial population in the given sputum sample, unlike in the indirect test, which can suffer from errors of selection when drug susceptibility test was set up from a primary culture. In the direct tests, resistance can be reported if adequate growth is seen on the drug slopes even when the plain medium is contaminated. Resistance could be detected with growth as low as 10 colonies and with total agreement with the result of the indirect test; however, it is recommended that such results be accepted provisionally and confirmed with indirect test on the subculture. The direct tests described here would serve the purpose well in countries with limited resources.

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