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*Original Article*

# RAPID ANTIBIOTIC SUSCEPTIBILITY TESTING OF *MYCOBACTERIUM TUBERCULOSIS*: ITS UTILITY IN RESOURCE POOR SETTINGS

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## Abstract

**Purpose:** To compare the rapid colorimetric nitrate reductase based antibiotic susceptibility (CONRAS) test performed on *Mycobacterium tuberculosis* isolates with the conventional method i.e., the proportion method. **Methods:** One hundred clinical isolates of *M. tuberculosis* were tested for susceptibility to isoniazid (INH) and rifampicin (RIF) by the conventional proportion method and CONRAS in Middlebrook 7H9 liquid medium enriched with growth supplements (MB7H9S). **Results:** The performance of the CONRAS test was evaluated using proportion method as the gold standard. The sensitivity (ability to detect true drug resistance) and specificity (ability to detect true drug susceptibility) of the CONRAS test to INH was 93.75 and 98.52% and for RIF it was 96.10 and 100% respectively. The mean time for reporting was 6.3 days and the test showed excellent reproducibility. The kappa (κ) value for INH was 0.92 and for RIF was 0.99, indicating excellent agreement between the two methods. **Conclusions:** CONRAS test is a rapid and reliable method of drug susceptibility for *M. tuberculosis.*

**Key words:** *M. tuberculosis, nitrate reductase test, proportion method, rapid drug susceptibility*

WHO declared tuberculosis (TB) a global emergency in 1993 due to the increase in associated morbidity and mortality.[1] India has been classified along with the sub-Saharan countries to be among those countries with a high burden of disease i.e., group IV countries.[2] The incidence of TB has been steadily increasing since the onset of the HIV epidemic, attributed to the increased risk of TB infection in HIV infected population.[3] Another obstacle to be confronted is drug resistance.

Although drug resistance was observed even in the early days of chemotherapy nearly 50 years ago, the current threat is due to the emergence of strains resistant to the two most potent anti-tuberculosis drugs i.e., isoniazid (INH) and rifampicin (RIF).[4] Mumbai is considered as a hot zone for multi-drug resistant tuberculosis (MDRTB).[5] Patients infected with MDRTB strains not only pose a threat to themselves but to the community as well. It is therefore imperative to detect drug resistant strains at the earliest so that appropriate therapy can be initiated which will reduce the morbidity and mortality in infected patients and also prevent the spread of multi-drug resistant strains in the community. Towards this, drug susceptibility testing has become an important aspect in the treatment and control of tuberculosis. Conventional drug susceptibility methods for *M. tuberculosis* take six weeks for the results to be obtained. This leads to loss of time and delayed initiation of proper treatment, resulting in the patient transmitting the drug resistant infection in the community. Advances in technology have led to the development of newer modalities of susceptibility testing based on automated systems or molecular diagnostic methods. These tests are however expensive. Hence, in resource poor settings, there is an urgent need for a rapid method of drug susceptibility, which can give early and reliable results and still be economical. More recently a number of low cost colorimetric assays have been described e.g., 3-(4,5-dimethlythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT assay), the resazurin microtiter plate assay and assays based on microscopic detection of cord like growth by *M. tuberculosis*.[6-8] But none of the above tests are without limitations e.g., INH can interfere with formazan production in MTT assay and can give falsely resistant results.[6] Mycobacteria other than *M. tuberculosis* can produce cord and string-like growth in liquid medium e.g., *M. avium, M. kansasii, M. bovis*.[8] The use of liquid medium in a microtiter plate can not only be a biological hazard but also result in contamination in between the wells and lead to false results.[7] An antibiotic susceptibility test based on nitrate reductase performed on Lowenstein Jenson medium (LJ) has also been described.[9,10] The results were comparable to the gold standard i.e., proportion method and the BACTEC 460 TB system and the results were available in seven to fourteen days in both the studies. The present study is aimed at detecting the utility of a rapid antibiotic susceptibility test based on the colorimetric nitrate reductase method i.e. CONRAS test as described elsewhere.[11]

## Materials and Methods

### *Strains*

The work was carried out in the Department of Microbiology at Seth GS Medical College and KEM Hospital, Mumbai. One hundred clinical isolates of *M. tuberculosis*

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identified using morphological and biochemical properties as per standard protocol[12] were selected over a period of one year i.e., from March 2004 to February 2005. All isolates were stored at -70ºC and sub cultured to obtain a fresh culture before use. A known nitrate positive (H37Rv) and a known nitrate negative standard strain (*M. avium intracellulare*) were used as positive and negative controls for the CONRAS test. The standard strains were obtained from the mycobacterial repository at the Central JALMA Research Institute, Agra. All strains were first confirmed for their nitrate reductase activity as per standard protocol.[12] A batch of 20 isolates was put in duplicates for the rapid antibiotic susceptibility test to assess the reproducibility of the test. The test was carried out in batches of 10 isolates at a time.

*Inoculum for CONRAS test*

A uniform bacterial suspension of 0.5McFarland and 1McFarland turbidity standard was prepared in Middlebrook 7H9 (Becton Dickinson) liquid medium supplemented (MB7H9S) with 0.5% glycerol, 0.05% Tween 80, OADC (oleic acid, albumin, dextrose and catalase - Becton Dickinson) and 850 µg/mL of sodium nitrate (Merck). This suspension was used as the inoculum for the CONRAS test.

*CONRAS test*

The CONRAS test was performed with 0.1 µg/mL of INH (Sigma) and 1 µg/mL of RIF (HI Media Laboratories) as described by Syre *et al*.[11] Briefly, for each strain tested, a panel of eleven tubes (round bottom 110 x 16 mm screw capped) were prepared [Table 1].

The readings were taken on day three, day five, day seven or day nine after incubation. The control tube was first visualized for evidence of growth. A 'no growth' was considered a valid test and then nitrate reduction test (NRT) was performed in the following order. The initial reading was taken on day three with the test first being performed on one of the 1 McFarland tubes (tube 3). If the color intensity developed was <4+, this tube was discarded and the remaining tubes were incubated further. On the other hand, if the color intensity in tube 3 was ≥ 4+, NRT was performed on one 1:5 dilution of 0.5 McFarland tube (tube 6). If the color intensity in tube 6 was ≥ 4+, NRT was performed on the antibiotic containing tubes along with the 1:500 dilution of 0.5 McFarland tube (tube 9). If on the other hand, the color intensity in tube 6 was <4+, the remaining tubes were incubated further for an additional two days and the tubes in which NRT was performed (tube 3 and 6) were discarded. The test is performed then on day five in a similar manner. When the color intensity in the drug free tubes was 1+ or 2+ on day five, the tubes were incubated further for four days and the NRT was performed on day nine instead of day seven.

The color intensity of the drug free and drug containing tubes was noted visually or by a spectrophotometer (Genesis 10 UV). A strain was classified as resistant when the color intensity of the antibiotic containing tube was ≥ 2 gradations above that of the drug free tube (1:500 dilution) for that antibiotic (Figs. 1 and 2) or when the absorbance reading at 570 nm of the drug containing tube was > 0.03 OD units above that of the optical density at 570 nm in the drug free medium (1:500 dilution).

*Nitrate reduction test*

The nitrate reduction test was performed by the addition of specific reagents i.e., 25 µL of 1:1 diluted hydrochloric acid (HCl), 50 µL of 0.2% sulfanilamide (Merck) and 50 µL of 0.1% N-1-naphthylethylenediamine dihydrochloride (Merck) in that order to the inoculum.[12] The development of pink color was read visually after five minutes. Additionally, for 50 isolates absorbance, reading was taken at 570 nm using a spectrophotometer. A small amount of zinc was added to detect true negative strains, which could not be detected by the above reagents. A reference standard for visual reading of the color intensity was prepared by serial two fold dilutions of 1:128 dilution of Sodium nitrite stock solution. (Fig. 3).[11]

**Proportion Method**

Antibiotic susceptibility testing was performed on LJ medium according to the standard protocol laid down by WHO.[13] The drug concentrations used were INH 0.2 µg/mL and RIF 40 µg/mL (critical concentration). The reading was taken on the 28th day and if a strain was susceptible it was further incubated and a second reading taken on the 42nd day as the final reading.

*Statistical analysis*

The results of the CONRAS test and proportion method were analyzed. Sensitivity and specificity was calculated using standard formulae. Kappa values (k) were determined to know

**Table 1: Panel of tubes used for CONRAS test**

| | | | | | | |
|---|---|---|---|---|---|---|
| MB7H9S | 1 ml | 1 ml | 1 ml | 1 ml | 1 ml | 1 ml |
| BS | 110 µl BS | 110 µl | 110 µl | 110 µl | 110 µl | None |
| Inoculum size | (0.5 McF) | (0.5 McF) | (1 McF) | (1:5 dilution of 0.5 McF) | (1:500 dilution of 0.5 McF) | None |
| Drug | INH | RIF | None | None | None | Control |
| Concentration | (0.1 µg/ml) | (1 µg/ml) | | | | |
| Tube number | 1 | 2 | 3,4,5 | 6,7,8 | 9,10 | 11 |

BS = Bacterial suspension, McF = McFarland, INH = Isoniazid, RIF = Rifampicin. The entire panel of tubes was kept for incubation at 37ºC.

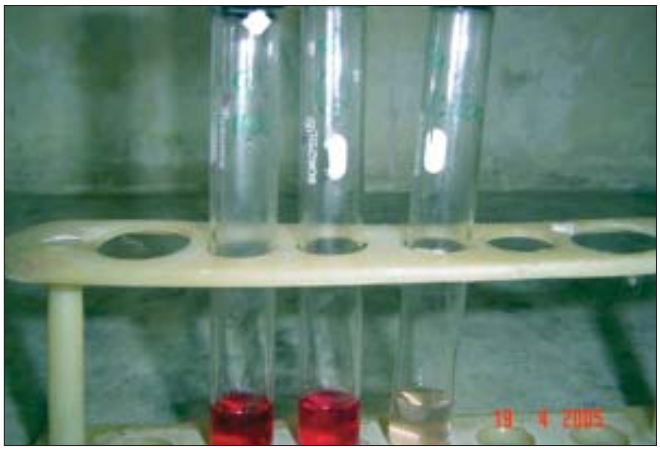

**Figure 1:** A resistant strain with the 1:500 tube showing a 2 + and the individual tubes of INH and RIF showing 6 +

**Figure 2:** A sensitive strain with the 1:500 tube showing a 1 + and the individual tubes of INH and RIF showing 2 +

the agreement between the two methods.

## Results

Of the 100 *M. tuberculosis* isolates tested by the proportion method, 58 strains were sensitive to both antibiotics tested, 16 strains were resistant to isoniazid alone, 10 strains were resistant to rifampicin alone and 16 strains were resistant to both the drugs i.e., these strains were multi-drug resistant (MDR).

One strain, which was detected as resistant by the proportion method, was reported sensitive by the CONRAS test for rifampicin and two strains that were detected as resistant by the proportion method were reported sensitive by the CONRAS test for INH. The level of agreement between the CONRAS test and the proportion method for INH was 97% and for RIF 99% (Table 2). The kappa (k) value for INH was 0.92 and for RIF 0.99 indicating excellent agreement. The sensitivity (the ability to detect true drug resistance) and specificity (the ability to detect true drug susceptibility) of the

**Figure 3:** A reference standard for visual reading of the color intensity was prepared by serial two fold dilution of 1:128 dilution of Sodium nitrite (NaNO2) stock solution (10 mM NaNo2). The standard tubes are graded from 1 + through 6 + (From left to right)

CONRAS test using the proportion method as the gold standard were 96.1 and 100% for RIF and 93.75% and 98.52% for INH respectively (Table 3).

*Reporting time*

The CONRAS test results were available for 8 isolates in three days, for 35 isolates in five days, for 45 isolates in seven days and for 12 isolates in nine days. Thus the average reporting time for the CONRAS test was found to be 6.3 days. It was observed that the strains that showed discordant results took a longer time to be reported (day nine). The proportion method took 42 days for the final result to be obtained.

*Reproducibility*

The reproducibility of the CONRAS test was assessed by putting twenty isolates in duplicates. All the isolates gave the same results.

*Costing*

The cost of the CONRAS test worked out to be Rs. 145/- per isolate inclusive of all the media, chemicals and reagents used. The cost of the conventional proportion method worked out to be Rs. 45/- per isolate. This was not inclusive of the expenses involved in salary and infrastructure required for performing the tests as these would be common and hence were not considered.

## Discussion

Multi-drug resistant tuberculosis (MDR-TB) is an emerging problem in our country; more so in the wake of the HIV epidemic with Mumbai being described as a hot spot for MDR-TB.[5] Although directly observed treatment short course (DOTS) can reduce the emergence of drug resistance,[14] it has been found that patients with initial resistance to INH and RIF, when treated with the standard DOTS, subsequently develop

**Table 2: Susceptibility to isoniazid and rifampicin by CONRAS test and the proportion method**

| | Susceptibility by both methods | Resistant by both methods | Susceptible only CONRAS test | Susceptible only proportion method | Agreement % |
|---|---|---|---|---|---|
| Isoniazid | 67 | 30 | 02 | 01 | 97 |
| Rifampicin | 74 | 25 | 01 | 00 | 99 |

**Table 3: Sensitivity and specificity of the CONRAS test for rifampicin (1 μg/mL) and for isoniazid (0.1 μg/mL)**

| | Proportion method | | | |
|---|---|---|---|---|
| CONRAS test | RIF resistant | RIF sensitive | INH resistant | INH sensitive |
| Resistant | 25 | 00 | 30 | 01 |
| Sensitive | 01 | 74 | 02 | 67 |
| Total | 26 | 74 | 32 | 68 |

Sensitivity for RIF and INH = 96.1 and 93.75% respectively, Specificity for RIF and INH = 100 and 98.52% respectively, RIF - Rifampicin, INH - Isoniazid

resistance to the other drugs also.[15] The increasing trends of MDR-TB calls for necessary amendments to be made in control programmes to tackle problems like multi-drug resistance. Therefore, drug susceptibility testing is a prerequisite, not only for the detection of MDR-TB but also for the initiation of appropriate therapy in such patients.

Conventional methods of drug susceptibility do not give early results and so lose their purpose in benefiting the person and the community at large. Therefore methods that can give us early and reliable antibiotic susceptibility test (AST) results have become the need of the hour. We evaluated a nitrate reductase based assay i.e., CONRAS in a liquid medium (MB7H9S) as a rapid and reliable antibiotic susceptibility method for INH and RIF and also determined its utility in resource poor settings.

More than 99% of *M. tuberculosis* strains produce nitrate reductase enzyme, which can reduce nitrates to nitrites. A few other mycobacterial species e.g., *M. smegmatis, M. kansasii, M. flavescens, M. fortuitum* also possess this enzyme. But these species can be differentiated using the morphological and biochemical tests.[12] The peak activity of nitrate reductase in LJ medium is found to be after three to four weeks of incubation but this time is considerably shortened by the use of a liquid medium i.e., Middlebrook 7H9S.[11]

Before performing the CONRAS test on test isolates, the test was performed on standard susceptible strain of H37Rv that gave acceptable results. The CONRAS test was evaluated against the conventional proportion method. The days on which the NRT was to be performed was based on the fact that nitrate reductase activity in liquid medium begins on the third day and is at its peak between five to nine days after incubation.[11] The average reporting time for CONRAS test was 6.3 days as against the 42 days for the conventional method. This is very much comparable with the results of the manual MGIT and the BACTEC 460 TB, which require the use of expensive equipment and a high running cost. The rapidity with which the test could be performed has also been reported in other low cost assays e.g., Resazurin microtitre plate assay, microscopic observation broth drug susceptibility (MODS).[7,8]

The overall agreement between the two methods was excellent for the individual drugs i.e., 97% for INH and 99% for RIF. The kappa (k) value for INH was 0.92 and for RIF 0.99 indicating excellent agreement. The CONRAS test was able to detect 30 out of the 32 strains that were INH resistant and 25 out of the 26 strains that were RIF resistant. For detecting MDR - TB, the major error i.e., reporting a susceptible strain as resistant, was 0.70% and the very major error i.e., reporting a resistant strain as susceptible was 5.1% with the CONRAS test. For individual drugs, the major and very major errors were 0 and 3.84% respectively for RIF while those for INH were 1.47 and 6.25% respectively. The sensitivity and specificity of the CONRAS test using the proportion method as the gold standard were 96.1 and 100% for RIF and 93.75 and 98.52% for INH respectively. This is marginally lower than those reported in literature.[6-9,11] This may be because the sensitivity of the CONRAS test depends on the metabolic activity of the organism, which is directly proportional to the measurable nitrate reductase activity of the organism. In case of the resistant strains it has been observed that their metabolic activity is low.[16] It is possible that these three strains could have had low levels of resistance and low metabolic activity. Molecular techniques for detecting genes conferring drug resistance (katG, rpoB) could have helped to resolve these discrepancies.[17] The high sensitivity of these tests, as evidenced by the results of the present study, makes them useful tools for detecting drug resistance and guiding choice of therapy. Further studies, which can compare the *in vitro* results with the clinical response, need to be undertaken. The results of such studies will further highlight the clinical usefulness of antibiotic susceptibility tests especially where long term follow-up of patients is required as in tuberculosis.

There was excellent agreement between the visual and the spectrophotometry results of the CONRAS test and hence we

could do away with the need for a spectrophotometer for recording further results. The minimum equipment required for performing the CONRAS test include a biological safety cabinet (BSC - Class II),[12] a vortex mixer and a 37ºC incubator. Some studies have pointed out that the color reaction of the nitrate test may be unstable and hence results must be read as soon as possible.[18] In our study, we did not encounter such a difficulty. The color reaction was stable and hence a number of tests could be performed at the same time. Nonetheless, certain precautions need to be taken while performing the CONRAS test in order to avoid contamination and false results. They are as follows:

a) All glassware must be thoroughly cleaned and autoclaved before being used.

b) All media, reagents, antibiotic stock solutions must be freshly prepared and dispensed in small aliquots to prevent contamination.

c) Only fresh cultures must be used (<14 days), as results are not consistent with old cultures.

d) The temperature of the incubator must be monitored daily.

e) The nitrate reducing capacity of each *M. tuberculosis* strain should be confirmed before proceeding with the antimicrobial susceptibility test.

Though the actual cost of the rapid test is almost thrice the conventional test, it is still within reach of the patient or national programme. The OADC supplement is the reason for this cost difference and if a cheaper enriching medium can be used which can support the growth of *M. tuberculosis*, then the cost of the test will reduce even further.

A recent study by Montoro *et al*, has recommended that the nitrate reductase test can be an inexpensive alternative for rapid detection of resistance for the first line drugs in low resource countries.[19] Our results are also very similar and we conclude that the CONRAS test is a rapid, reliable and a low cost method for drug susceptibility testing of *M. tuberculosis*.

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