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Comparison of REMA with Agar Proportion method GeneXpert MTB/RIF, and HAINE assay for drug resistance among M. TB isolates

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Abstract:

Introduction: Rapid identification of drug susceptibility profiles of M. tb facilitates early treatment and improve treatment outcomes, prevent amplification of resistance and reduce the transmission of DR-TB.

Materials& Methods: In this prospective study,100 M.tbclinical isolates (grown on MGIT and that are also positive by the Gene Xpert M.TB/RIF) were analysed, susceptibility to INH & Rifampicin by a Resazurin based Microtiter Assay, GeneXpert, HAINE Line probe assay and gold standard Agar Proportion method. The results of all methods were compared and analysed. The standard M. tbstrain, H37Rv ATCC strain was used as a quality control in all the assays.

Results: Among the 100 M. tbisolates, 24 (24%) isolates were shown to RIF resistant by the Gene expert MTB/RIF. The colonies of 16/24 (66.7%) of the RIF resistant isolates were rough with irregular edges, while the rest had smooth and regular edges. On the Resazurin assay, the 20/24 (83.3%) RIF resistant isolates had a MIC ranging between 0.25 to 1 µg/ml for INH and 0.5 to 2 µg/ml for RIF. On the HAINE assay the 24 RIF resistant isolates, all the 24 (100%) isolates had a mutation of the RPOB gene; while14/16 (58.3%) showed mutation also of the KATgenes.

Discussion: Among the 3 assays, the results of Gene Expert and HAINE assay for the RPOB genewere similar. However, the latter could identify 14 isolates with mutation of the KAT gene. The Resazurin assay was technically challenging.

Conclusions: REMA proved to be cost effective and helpful in the rapid detection of drug resistance among the M. TBisolates.

Keywords: REMA compared to APM, Gene Xpert, Haines

Introduction

Tuberculosis (TB) is among the most common infectious diseases and frequent cause of death world-wide. An estimated one third of the world's population is infected with Mycobacterium tuberculosis (M.tb).It is estimated that 11 cases per 100,000 population develop active pulmonary smear positive TB annually, while 24 per 100,000 develop all types of TB annually according to World Health Organisation (WHO) report in 2010.[1] Multidrug resistant (MDR) Mycobacterium tuberculosisstrains have been observed around the world, with high mortality rates particularly among HIV-positive patients.[1,2]MDR TB is now also emerging in HIV-negative populations and in high-risk populations such as medical staff. Several rapid and accurate M. tb diagnosis have been developed, but some of them are high cost, require technicality, need sophisticated equipment with all-time electric power source, making their use difficult in low-income countries.[1,3] MDR Mycobacterium tuberculosis isolates present a major challenge for tuberculosis control programs. Rapid diagnosis of MDR patients is nevertheless necessary to avoid the spread of MDR strains.[1]

Calorimetric assays employing oxidation-reduction indicators for DST have been previously used with mycobacteria. Drug susceptibility testing (DST) with Lowenstein-Jensen (LJ) medium or Middle-brook agar requires 3 to 6 weeks to obtain results.[1,4] Critical drug concentrations for second-line drugs have not been completely established. A simple ,rapid and inexpensive colorimetric method based on the oxidation-reduction indicators Alamar blue have been recently used for determining MICs of first-line drugs in DST of Mycobacterium tuberculosis.[5] Resazurin, an oxidation-reduction indicator, has been used to assess viability and bacterial contamination and to test for antimicrobial activity. Since Alamar blue has been recently identified as resazurin in cell cytotoxicity studies , it was standardized and evaluated a micro plate method (Resazurin Microtiter assay (REMA) plate) which uses the reduction of resazurin for DST to INH and RIF in clinical isolates of Tuberculosis.[6] A majority of these cell wall genes also contribute to the in vivo survival of M. tb, consecutively forming a barrier for drug action and leading to drug resistance.

Currently, the two rapid assays recommended by the WHO are GeneXpert and line probe assay. GeneXpert and line probe assay can be used for rapid detection of mutation resulting in rifampicin resistance, while LPA can also detect mutation related isoniazid resistance.[7,8]These rapid molecular methods enable early detection and treatment of cases, which is essential in preventing MDR-TB from progression to XDR-TB or, even worse, reaching the TDR-TB level.[7,8,9]

Aim/purpose

Comparison of simple method like Resazurin assay with Agar Proportion Method (Gold standard), genotype assays GeneXpert M.TB/RIF and Haines Line probe assay

Materials and Methods

- This is a Prospective study (sept 2020-sept 2022) performed in the Department of Microbiology at Kamineni Academy of Medical Sciences and Research Centre, Hyderabad, Telangana, India.

- 100 M. tbclinical isolates (grown on MGIT and/or LJ and that are also positive by the GeneXpert M. TB/RIF) were analysed by

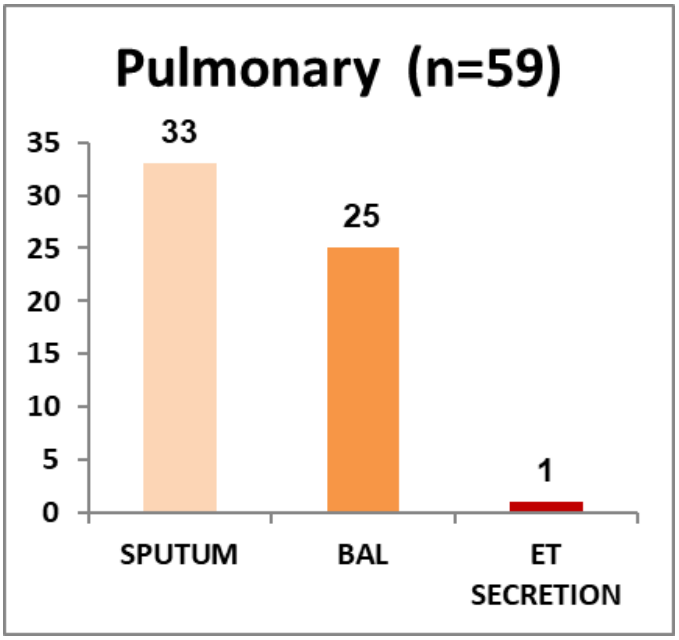
Drug susceptibility using 1% agar proportion method

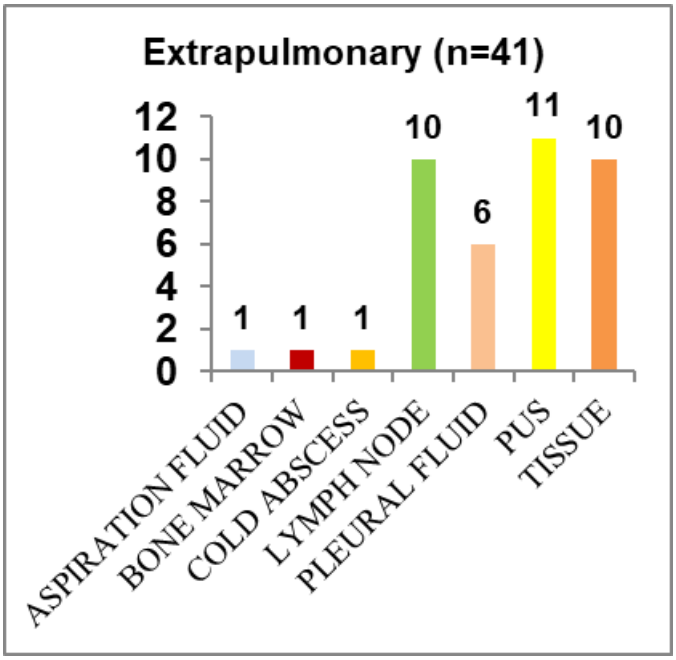
Susceptibility of TB isolates was determined using gold standard Agar Proportion Method(APM) on Middlebrook 7H10 media for rifampicin and isoniazid. 100 microliters from a MGIT positive culture was inoculated into quadrant of 7h10 DST agar plate and incubated for 20 days. Growth of more than 1% on anti-tubercular drug containing quadrant when compared to drug free growth control was taken as resistant to tested anti-tubercular drug.

- Susceptibility to INH & Rifampicin was done by a Resazurin Microtiter Assay (REMA) and HAINE Line probe assay.
- The standard M. tbstrain, H37Rv ATCC strain was used as a quality control in all the 4 assays.

The results of all methods were compared and analysed.

Fig1: Distribution of samples from which MTB were isolated.





Resazurin Microtiter assay (REMA) plate Method[10,11]

The inoculum was prepared from fresh LJ medium in 7H9-S broth, adjusted spectrophotometrically to a no.1 McFarland tube standard, and further diluted 1:10 in 7H9-S broth for the test. The REMA plate assay was performed by using 100 µl of 7H9-S broth was dispensed in each well of a sterile flat-bottom 96-well plate and serial two-fold dilutions of each drug were prepared directly in the plate. One hundred microliters of inoculum was added to each well. A growth control and a sterile control were also included for each isolate. Sterile water was added to all wells to avoid evaporation during the incubation. The plates were covered, sealed in a plastic bag, and incubated at 37ºC under a normal atmosphere. After 7 days of incubation, 30 µl of resazurin solution was added to each well, and the plate was re-incubated overnight. A change from blue to pink indicates reduction of resazurin and therefore bacterial growth. The minimal inhibitory concentration (MIC) was defined as the lowest concentration of the drug that prevented this change in colour. A change in colour of growth control well to pink indicated the proper growth

of the isolate and no change incolour of sterile control well indicated absence of contaminants.

Fig 2: REMA plate method assay for RIF & INH resistant isolates with MIC rangingbetween 0.5 to 2 µg/ml for RIF & 0.25 to 1 µg/ml for INH

Line Probe Assay (LPA)[12]

It is based on PCR line probe assay. Identifies Mycobacterium tuberculosisand detects RIF and INH resistance due to mutations in rpoß,and bothinhAand kat G genes in a day. The method involved three processes: DNA extraction, multiplex PCR amplify cation, and reverse hybridization. The DNA strip was removed from the tube and marked as per the number of samples. It was then added to each well containing 20 µl of corresponding amplified DNA sample with coloured part facing up. The well was placed in the incubator and hybridization procedure was initiated. Hybridization occurred by pre-warming the hybridization buffer to 45 ºC in water bath for 15 min in the incubator machine. Denaturing solution, 20 µl was pipetted to each of the tray that was used, and then 1 ml of rinse solution added per well and incubated for 1 min. The well was removed and rinsed with a rinse solution. One millilitre of the conjugate was added into each well, then incubated for 30 min, removed and washed with rinse solution. Finally 1 ml of the substrate was added into the well and incubated for 10 min and then washed twice with distilled water. The strips were then left to dry and results scanned and interpreted as either Mycobacterium tuberculosisdetected, resistant, sensitive or invalid.

Results

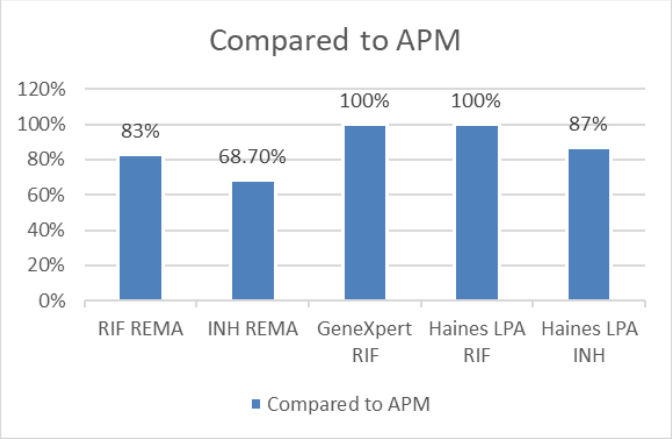
100 isolates from MGIT tube of patients from various clinical samples picked and subjected to APM, GeneXpert, LPA, spot phenotype and Resazurin Microtiter plate assay. Out of 100 M. tbisolates 59 were pulmonary and 41 were extra pulmonary. Among pulmonary, Sputum samples were more in number (n=33, 55.9%), BAL (n= 25, 42.3%), ET (n=1, 1.6%). Among extra-pulmonary, pus was predominant (n=11,26.8%), Lymph node and Tissue (n=10,24.3%) Pleural fluid (n=6, 14.6 %). Out of 100 samples, M: F ratio showed 87:13, Males were more in number. Among the 100 M. tbisolates, APM detected 24 isolates as rifampicin resistant and 16 isolates as isoniazid resistant, all 24 were detected as RIF resistant by the GeneXpert MTB/RIF. On the Resazurin micro titre plate assay (REMA), 20/24 (83.3%) RIF & 11/16 (46%) INH resistant isolates had a MIC ranging between 0.5 to 2 µg/ml & 0.25 to 1 µg/ml respectively.

HAINE assay the 24 RIF resistant isolates, all the 24 (100%) isolates had a mutation of the RPOB *gene*; while14/16 (87.5%) showed mutation also of the KAT gene. Fig 3. The REMA detected 20 rifampicin resistance out of 24 resistant isolates. Overall sensitivity, specificity, positive and negative predictive values were 85.71%, 98.7%, 96% and 95% respectively for detecting

rifampicin resistance. Whereas out of total 16 INH resistant isolates by APM method, REMA detected 11/16 (46%). The sensitivity, specificity, positive and negative predictive values were 68.75%, 98.82%, 91.6%, 94.3% respectively.

The genotypic methods show similar results as APM method. The line probe assay did not detect 2 isolates as INH resistant.

Fig 3: Comparison of drug resistance detection in various methods to gold standard APM method



Discussion

The purpose of TB drug susceptibility is to know resistant strains that lead to treatment failure and relapse. The GeneXpert and Line Probe assay are faster methods of detection but come with some limitations such as only RIF detection in case of Xpert, expertise requirement in case of line probe assay with added high cost per test.

The REMA assay on the other hand has short TAT of 8 to 9 days, low cost, easy to interpret and no special equipment required. Disadvantages are high chances of aerosolization, but this can be countered by processing the test in biosafety cabinet. Though low cost and faster it is labour intensive, requiring all individual drugs and dye preparation and micro titre plate inoculation.

The present REMA study showed comparable sensitivity and specificity to that of APM and genotypic methods, GeneXpert and HAINE Line probe. Which is similar to previous studies Jaglal P et al and Khalifa RA et al. There was over 90% agreement between REMA and other reference methods.[4,13]

Limitations

In our study we only compared with first line anti-tubercular drugs, the second line drugs were not used.

Conclusion

The molecular assays, Gene Xpert and HAINE prove to be performing better than the phenotypic assays in the detection of drug resistance among the M.tb isolates.

Rapid identification of drug susceptibility profiles of M. tb facilitates early treatment and improve treatment outcomes, prevent amplification of resistance and reduce the transmission of DR-TB

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