Drug Resistance Detection in Mycobacterium Tuberculosis

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Abstract: *MDR cases of tuberculosis are increasing and they are difficult to treat. This study was conducted to assess prevalence of drug resistance in Mycobacterium tuberculosis. Study comprises of 67 sputum specimen received for Mycobacterium tuberculosis (MTB) culture. Culture was done in Middlebrook broth using BacT Alert 3D automated culture system. The study period was from Dec 2018 to Nov 2019. Out of these MTB was grown in 31 (46.27%) cases. The first line sensitivity testing was done in liquid culture media (BacT Alert3D) against Isonizid, Rifampicin, Ethambutol, Streptomycin. Isonizid and Rifampicin resistance was seen in 3 (9.68%) cases. Only Isonizid resistance was seen in 9 (29.03%) cases. This indicates that multidrug resistance is present to the tune of 9.68 % while monoresistance to isonizid is more common in 29.03%. Monoresistance to rifampicin was not observed. Resistance to ethambutol and streptomycin was not observed in the present study. The advantage of doing Drug susceptibility testing in liquid media over solid media is speed and the results can be obtained within 15 days as compared to over 4 weeks when tested in solid media. Rifampicin resistance can be considered as a marker for labeling the strain as MDR.*

Keywords: Mycobacterium tuberculosis, Multi drug resistance (MDR), Bact-alert 3D, Rifampicin, Isoniazid.

1. Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, remains one of India's biggest health problems. Every year, India reports over 2 million TB cases. With the emergence of severe forms of drug-resistant TB and concerns about TB drug shortages, there is much work to be done to control the epidemic(1). The worldwide prevalence of TB was 9.6 million in 2014, with 1.5 million deaths(2). Pulmonary infection is the most prevalent contagious clinical form of tuberculosis, and is therefore of prime public health importance(3).Fast and effective microbiological diagnosis is essential to control the spread of TB.

The BacT/Alert 3D system is a fully automated liquid culture system which allows the growth and detection of mycobacteria. It is a rapid and sensitive method for recovery of mycobacteria from clinical specimens using an N-acetyl-L-cysteine-NaOH decontamination method (4). However, the emergence of resistant strains, including multi-drug resistant (MDR) and extremely drug resistant (XDR) strains has posed a significant challenge. Though advances in drug therapy have been limited, TB control has greatly benefited from the advent of newer diagnostic tests including use of liquid culture media. Culturing *Mycobacterium tuberculosis* remains the gold standard for the laboratory diagnosis of pulmonary tuberculosis.

Multidrug-resistant tuberculosis (MDR-TB) is defined as disease due to *Mycobacterium tuberculosis* that is resistant to isoniazid (H) and rifampicin (R) with or without resistance to other drug(5). The emergence of multidrug-resistant tuberculosis (MDR-TB) and, more recently, extensively drug-resistant TB (XDR-TB) is widely considered a serious threat to global TB control. Detection of drug resistance in Tuberculosis by conventional disc diffusion is not possible due to slow speed of growth of Mycobacteria. Therefore dilution methods have been used(6).

In the present study the drug susceptibility testing was standardized in liquid culture media using the automated system of BacT Alert 3D (bioMerieux) and its utility was assessed for clinical use.

2. Literature and Review

Multidrug-resistant TB (MDR TB), i.e. resistance to at least isoniazid (Inh) and rifampin (Rif), and extensively drug-resistant TB (XDR TB), i.e. MDR plus resistance to amikacin, kanamycin or capreomycin and a fluoroquinolone, are the most problematic forms of resistance because treatment options are limited and the second-line drugs used for therapy are more toxic, less effective, more expensive, and must be administered for a longer period of time than standard first-line drug therapy(7). Conventional culture and DST on solid media is a slow process, and in high income, low-incidence countries these systems have been supplemented (or replaced) by automated liquid culture systems. The World Health Organization now recommends expanded use of liquid culture systems in resource constrained settings.

Seoung-Cheol Kim and co authurs evaluated the performance of the BacT Alert 3D system, a liquid culture system, for mycobacterial culture and drug susceptibility testing by comparison with mycobacterial culture using LJ medium and drug susceptibility testing with M-KIT plates in the situation of a high MDR-TB and XDR-TB burden. In our study population, the prevalence of MDR-TB and XDR-TB in our pool of isolates were 6.0% and 2.3%, respectively (8).

Increase in multidrug resistance from 4.7 to 19.8 per cent in the past 13 years, as observed by Reena Raveendranand freinds, needs to be noticed. In the present scenario of increasing prevalence of MDR-TB and lack of availability of many second line drugs, screening with culture and drug susceptibility testing should be recommended for all smear

positive pulmonary patients. The WHO policy guidelines[31] for the use of new rapid molecular based techniques for early detection of MDR-TB may help the high-burden countries in identifying and treating patients of MDR-TB quickly.(9)

3. Material & Methods

The study period was from May 2018 to Nov 2019. Study comprises of 67 sputum specimen received for Mycobacterium tuberculosis culture. After decontamination Culture was done in Middle brook broth using Bac T Alert 3D automated culture system.

Decontamination using Sodium hydroxide (modified Petroff) method:(10)
Sputum (at least 2 ml, not more than 5 ml) was transferred into a centrifuge tube. Equal volume of 4% NaOH and citrate was added. Cap was tightened cap and solution was vortexed and pinch of NALC (*N*-acetyl-*L*-cysteine) powder was added, mixed and allowed to stand for 15 minutes at room temperature. The tube was filled to within 2 cm of the top (e.g. to the 40-ml mark on the tube) with phosphate buffer, vortexed, mixed and centrifuged at $5000g$ for 15 minutes. The supernatant was carefully poured off into a discard can containing 5% phenol or other disinfectant. The deposit was resuspended into MP bottle of BacTalert.

In the BacT/Alert MB® (bioMérieux, Craponne, France), growth is inferred from increasing CO2 tension in a bottle. Once the bottle is flagged positive by the system, the bottle is removed, small amount of fluid is drawn using the syringe, ZN smear is prepared and Mycobacterium species is confirmed. *M. tuberculosis* was confirmed by TB Antigen MPT64 Rapid test. (SD Bio Line, Standard Diagnostics Inc.).

Drug susceptibility testing was done on the isolated strains of *M. tuberculosis*.

The bottle when flashes positive has $10^6 - 10^7$ CFU/mL which is equivalent to 1 McFarland.

Drug Susceptibility Testing: Drug susceptibility was done as follows (11)

Inoculum
To perform the sensitivity for the first line drugs, 6 bottles were used as follows:
1) Growth Control 2) 1% of Growth Control 3) Streptomycin 4) Isoniazid 5) Rifampicin 6)Ethambutol Inoculum of 0.5 ml of 1McF of Mycobacterium growth was added to Growth control bottle. (Bottle no 1)

From separate bottle 0.1 ml of media was discarded and replaced with equal volume of 1 McFarland growth. Now this bottle became 1% (diluted as 100 times) and from this bottle 0.5 ml of inoculum was added to the 1% GC bottle. (Bottle no. 2)

Steps to perform DST
The following concentrations of drugs were prepared:
Streptomycin (S): 20 µg/ml (Solvent: DW)

Isonizid (I): 2 µg/ml (Solvent: DW)
Rifampicin (R): 20 µg/ml (Solvent: DMSO)
Ethambutol (E): 100 µg/ml (Solvent: DW)

The drugs are added to the respective bottles with a volume of 0.5 mL so that the final concentrations in the bottles are as follows:
Streptomycin (S):1 µg/ml
Isonizid (I): 0.1 µg/ml
Rifampicin (R): 1 µg/ml
Ethambutol (E): 5 µg/ml

Reconstitution Fluid (0.5 ml)was added to the GC bottle also.

The 1 McF primary growth in oculum was added to all drug containing bottles. The rubber septum was cleaned with sterilium and kept in BacT/ALERT 3D equipment for at least 12 days.

Interpretation
The bottle flashing positive before 1% GC bottle with pure growth (confirmed by ZN) should be considered as Resistant and rest as Sensitive which comes positive after 1% GC bottle. The 1% bottle should flash positive in 12 days or else the test should be repeated.

4. Results

Out of 67 sputum liquid cultures, M. tuberculosis was grown in 31 cases with the culture positivity of 46.27 per cent. All the strains were confirmed to be M. tuberculosis by TB Ag MPT64 Rapid test.

The Drug Resistance testing was consistent in all 31 samples. The bottles became positive from 7 to 12 days. The average duration of resistance detection was 9 days. There were no inconsistent results. Isoniazid and Rifampicin resistance was seen in 3 (9.68%) cases. Only Isoniazid resistance was seen in 9 (29.03%) cases. This indicates that multidrug resistance is present to the tune of 9.68 per cent while monoresistance to Isonizid is more common in 29.03%. (Table 1).

Table 1: Showing Resistance pattern of M. tuberculosis

INH + RIFAMPICIN Resistance	3	9.68%
ONLY INH Resistance	9	29.03%
ONLY RIFAMPICIN Resistance	0	0.00%

In any of the 31 cases resistance was not detected to Ethambutol and Streptomycin.

5. Discussion

Multi Drug resistance is a problem in managing tuberculosis cases. Detection of drug resistance in tuberculosis is an important step in control of tuberculosis. In India, the prevalence of multi drug resistance as reported by WHO is 9.6%(12) and detection of drug resistance is important in order to detect these cases and treat them properly so as to have effective control of spread of disease. In the present study also MDR *M. tuberculosis* was detected in 9.68% cases. Resistance to ethambutol and streptomycin was not

observed in the present study. The agreement was lower for STR and EMB than for RIF and INH. (13) Isolated INH resistance was observed in 29.03% strains in the present study. Resistance to INH has also been reported to be high in other studies.(14), (11).

The drug susceptibility testing by conventional solid media like Lowenstein Jensen Medium will take about three to four weeks. While susceptibility testing using liquid culture media in automated system is fairly rapid, easy to perform and feasible in every set up where mycobacterial culture is done.

6. Future Scope

The technique being rapid and standardized, the resistance testing to second line drugs used for treatment of MDR strains can be performed and the data in this regard can be generated. Once standardized, this method can be used for routine testing of resistance to first as well as second line drugs also. If any potential new drug has to be tested, the same technique may be used with due modifications as required.

References

[1] Promoting Affordable and Quality Tuberculosis Testing in India. Pai, Madhukar. 2013, Journal of laboratory physicians, Vol. 5 (1), pp. 1-4.

[2] Evaluation of a liquid culture system in the detection of mycobacteria at an antituberculosis institution in China; A retrospective study. Ping Zhao, Qin Yu, Liang Chen, and Man Zhang. 2016, Journal of international medical resarch, Vol. 44 (5), pp. 1055-1060.

[3] Evaluation of a diagnostic algorithm for smear-negative pulmonary tuberculosis in HIV-infected adults. Saranchuk, P., Boulle, A., Hilderbrand, K., Coetzee, D., Bedelu, M., van Cutsem, G. 2007, South Africa medical journal, pp. 517-523.

[4] Evaluation of BacT/Alert 3D Liquid Culture System for Recovery of Mycobacteria from Clinical Specimens Using Sodium Dodecyl (Lauryl) Sulfate-NaOH Decontamination. A. Carricajo, N. Fonsale, A. C. Vautrin, and G. Aubert. 2001, Journal of clinical microbiology., pp. 3799-3800.

[5] Diagnosis and notification of multidrug resistant TB . (WHO), World Health Organization. Retrived 7 Decenmber 2016.

[6] Diagnosis of multidrug-resistant tuberculosis and extensively drug-resistant tuberculosis: Current standards and challenges. Giovanni Battista Migliori, Alberto Matteelli, Daniela Cirillo and Madhukar Pai. 2008, Canadian journal of infectious diseases and medical microbiology, Vol. vol 19, pp. 169-272.

[7] An Integrated Approach to Rapid Diagnosis of Tuberculosis and Multidrug Resistance Using Liquid Culture and Molecular Methods in Russia. Yanina Balabanova, Francis Drobniewski,Vladyslav Nikolayevskyy and et al. 2009, open acess plos one, Vol. vol 4.

[8] Performance of the BacT Alert 3D System Versus Solid Media for Recovery and Drug Susceptibility Testing of Mycobacterium tuberculosis in a Tertiary Hospital in Korea. Seoung-Cheol Kim, Bo-Young Jeon,. 2016, Tuberculosis and respiratory diseases, Vol. vol 4, pp. 282-288.

[9] Multidrug-resistant pulmonary & extrapulmonary tuberculosis: A 13 years retrospective hospital-based analysis. Reena Raveendran, Jaswinder Kaur Oberoi, and Chand Wattal. 2015, Indian journal of Medical research, Vol. vol 142, pp. 575-582.

[10] Decontamination of sputum for longer time in sodium hydroxide for isolation of Mycobacterium tuberculosis. P.SatapathyD.DasB.N.MurmuS.K.Kar. 2014, International journal of Mycobacteriology, Vol. vol 3, pp. 290-292.

[11] Standardization of first and second - line Antitubercular Susceptibility testing using Bact Alert 3D System- A report from Tertiary care centre in India. Deepthi Nair, Malini R. Capoor, Deepti Rawat, Lakshmi Srivastav and Pushpa Aggrawal. 2009, The Brazilian Journal of Infectious diseases, Vol. vol 13, pp. 422-426.

[12] WHO.Global Tuberculosis Report. s.l. : www.who.int>tb>publication>Global Report.

[13] Comparison of Mycobacterium tuberculosis drug susceptibility using solid and liquid culture in Nigeria. Lovett Lawson, corresNanmdi Emenyonu, Saddiq T Abdurrahman, Juliana O Lawson, Gertrude N Uzoewulu, Olumide M Sogaolu ane et al. 2013, Vol. vol 6, pp. 215-219.

[14] Prevalence of Drug resistance among M. tuberculosis isolates in Indore (MP). Chitnis, Hemrani N.and D S. 2002, Indian journal of Medical Microbiology, Vol. vol 22.

[15] New Vision for Revised National Tuberculosis Control Programme (RNTCP): Universal access-"Reaching the un-reached". achdeva KS, Kumar A, Dewan P, Satyanarayana S. 2013, Indian journal of Medical research, pp. 135-690.

[16] Evaluation of BacT/Alert 3D Liquid Culture System for Recovery of Mycobacteria from Clinical Specimens Using Sodium Dodecyl (Lauryl) Sulfate-NaOH Decontamination. A. CARRICAJO, N. FONSALE, A. C. VAUTRIN, AND G. AUBERT. Oct 2001, JOURNAL OF CLINICAL MICROBIOLOGY, Vol. vol 39, pp. 3799-3800.

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