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Analysis of *Mycobacterium tuberculosis* Drug Susceptibility to Extended Spectrum β - Lactam Antibiotics by Nitrate Reductase Assay

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Abstract

Background and Objectives: ESBLs are the lactam antibiotics hydrolyzing enzymes playing major barriers in employing lactam antibiotics to treat TB infection. In this study, we evaluated β-lactam antibiotics either pure or in combination with clavulanic acid for their Mycobacterium tuberculosis growth inhibiting potential by nitrate reductase assay which utilizes the detection of nitrate reduction as an indication of growth.

Methods: 100 AFB-Positive sputum samples collected from different clinical settings in district Saharanpur (U.P.) of India and H37Rv control strain was used as a control. Clinical isolates of M. tuberculosis were tested for six β-lactam antibiotics either pure or in combination with clavulanic acid by Nitrate Reductase Assay (NRA) and were compared with standard proportion method. The bacteria were inoculated on Lowenstein-Jensen (LJ) medium with ESBL drugs and potassium nitrate was incorporated. After incubation for a period of 10 days to 20 days, the mycobacterial growth was detected by color change due to nitrate reduction when reagents were added.

Results: All the clinical isolates of M. tuberculosis showed resistance for piperacillin, carbenicillin, mezlocillin, ticarcillin, ceftriaxone, and cefotaxime when used alone but the growth of M. tuberculosis isolates was significantly decreased when used in combination with the clavulanic acid. Complete agreement (100%) was found for β-lactam antibiotics with clavulanic acid. In contrast to proportion methods usually which takes 4weeks to 6 weeks LJ Culture method, the drug susceptibility results by NRA could be observed within 10 days to 20 days.

Interpretation and Conclusion: The ESBL resistance in all the M. tuberculosis clinical isolates was proved and significant growth inhibition of M. tuberculosis was observed when β-lactam antibiotics in combination with the clavulanic acid. The results of susceptibility were obtained as early as 7 days to 10 days by NRA. This also proved that NRA could be an appropriate alternative to testing M. tuberculosis drug susceptibility in a clinical setting with only very basic testing facilities.

Keywords: ESBL; β-lactam antibiotics; Drug susceptibility; Mycobacterium tuberculosis; Nitrate reductase assay

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Introduction

Mycobacterium tuberculosis, causing the disease tuberculosis (TB has been the leading cause of death worldwide. As per the World Health Organization (WHO) report in 2009, there were an estimated 9.27 million incident cases of TB and about 1.8 million deaths occurred due to tuberculosis in 2007 [1]. In 2016, there were an estimated 10.4 million new (incident) TB cases and approximately 1.8 million people died globally due to TB in the year 2015 [2].

Today, Multi-Drug Resistance (MDR) is a growing clinical problem, with strains of *M. tuberculosis* exhibiting resistance to 11 or more antimicrobial agents [3,4] and also with upsurgence of human immunodeficiency virus (HIV) infection/Acquired Immunodeficiency Syndrome (AIDS) [5,6] there is an urgent need of an alternative treatment strategy for the tuberculosis disease. Thus, it has become necessary to identify alternative treatment regimens including the older classes of antibiotics such as the β-lactams which might be effective in the clinical settings. Therefore, this study was carried out to evaluate the potential of β-lactams antibiotics as an alternative drug regimen for treating tuberculosis.

Further, there is a lack of proper treatment in the underprivileged area and distant villages due to insufficient control procedures and a long time taken by laboratory to test drug susceptibility and identify *M. tuberculosis* isolates [7,8]. The standard methods such as proportion, absolute concentration and the resistance ratio method for *M. tuberculosis* drug susceptibility testing is very time-consuming as they depend upon culture growth [9]. However, the quicker methods such as BACTEC require expensive instrumentation and highly skilled technicians, therefore is not feasible in most resource-poor settings [10]. Thus, a rapid, reliable and inexpensive method is needed for drug susceptibility testing of *M. tuberculosis*. Therefore, present study has been carried out to evaluate the efficiency and reproducibility of Nitrate Reductase Assay (NRA) for mycobacterium susceptibility testing. NRA is based on the ability of *M. tuberculosis* to reduce nitrate to nitrite, this reduction can be detected by the production of a color change in presence of specific reagents [11].

Materials and Methods

Primary isolation of *M. tuberculosis* and confirmation of β-Lactamases by Nitrocefin test

A total of 250 AFB positive clinical isolates of *M. tuberculosis* were collected from different clinical settings of District Saharanpur, Uttar Pradesh, India. The culture of the positive strain of *M. tuberculosis* H37Rv was used as a control. The chemicals and kits used were of standard companies and the Bio-Safety Level-3 (BSL-3) lab at CSIR (IGIB), New Delhi, India was used for the experiments on *M. tuberculosis*. The experiments were conducted at the Department of Zoology, M.S. College Saharanpur, under CCS University Meerut, U.P, India. The materials used and the methodologies adopted are as follows.

Isolation of *M. tuberculosis* clinical isolates

Out of AFB positive 250 MTB clinical isolates, 100 clinical isolates which grew successfully on L. J. Culture Slants and confirmed for their belonging to *M.tuberculosis* by Nitrate Reduction Test Niacin Production Test, Polymerization Chain Reaction (PCR) and confirmed positive for the production of β-lactamases as described in our previous publication [12] were selected for this study. The H37Rv (ATCC 25618) strain of *M. tuberculosis* was used as a positive control for the experiments.

Proportion method for drug susceptibility

Drug susceptibility of all 100 isolates to Carbenicillin, Mezlocillin, Piperacillin, Ticarcillin, Cefotaxime, and Ceftriaxone was performed by the standard method [13]. Drug MIC was determined by using 4 mg moist weight per milliliter (ml) of culture suspension by suspending 1/3rd loopful of 2 weeks to 3 weeks old culture, grown on L-J medium, in 1 ml of sterile distilled water and vortexing it to obtain a uniform suspension. The aggregates or undissolved particles in the suspension were allowed to settle at room temperature. The MTB colonies from 3 weeks to 4 weeks old culture were scraped with an inoculation loop and bacterial suspension was made in sterile distilled water, vortexed and density was maintained with McFarland opacity tube No.1. Dilutions of 10^{-2} and 10^{-3} were made and inoculated on both the control and drug containing media and incubated at 37°C. LJ media incorporated with drugs in various concentrations and also in combination with clavulanic acid and plain LJ medium for control were prepared. After incubation for 28 days and 40th day, the first and second readings were respectively taken. For calculating the percentage Resistance (R) below formula was used

R(%) = (No. of colonies on drug media) / (No. of colonies on control media) × 100

If R = >1 percent, the isolate was taken as resistant

Nitrate reductase assay for drug susceptibility

NRA was performed by standard procedure as described earlier [11,14]. Briefly, the tubes incubated in triplicate at 37°C for 14 days and 0.5 ml solution of three reagents (25 µl of concentrated HCl, 50 µl of 2% sulphanilamide and 50 µl of 1% n-1-naphthyl-ethylenediamine dihydrochloride) was added to control tube with no drug after 7 days of incubation. The color change to pink confirms a positive MTB growth and also a positive nitrate test. Then tubes with drugs were tested. An MTB isolate was considered resistant if there was a color change, from pink or deep red to violet, in the drug tube under investigation greater than in the 1:10 diluted growth control on the same day. If the tubes did not show any color change and remain the same, these were further incubated for 10 days and for 14 days. Mc Nemar's test was used for statistical analyses of data.

Results and Discussion

An apparent resistance was seen against β-Lactam antibiotics both by proportion and Nitrate reductase assay for carbenicillin, mezlocillin, piperacillin, ticarcillin, cefotaxime, and ceftriaxone which either showed inhibition of *M. tuberculosis* growth at a very high concentration or no inhibition was observed. However, when β-Lactam antibiotics were used in combination with clavulanic acid (2:1 ratio) a significant decrease in the MIC for these drugs was observed (Table 1). The results of β-Lactam antibiotics were in accordance with the findings described by earlier researchers [15,16,17]. The nitrate reductase assay was compared with the proportion method as a standard for susceptibility testing. The results of this comparative analysis have been presented in the Table 2. The results showed that there was no significant difference ($P > 0.05$ for all the drugs) between the values obtained for NRA and proportion method. An excellent agreement was observed between the NRA and proportion method results for all the beta-lactam drugs *i.e.*, 100% agreement for carbenicillin, mezlocillin, cefotaxime, and ceftriaxone while 99% agreement for piperacillin and ticarcillin.

In earlier studies carried on conventional anti-tuberculosis drugs, 98% to 100% agreement of susceptible strains between nitrate reductase assay has been found by Paniotov et al. [18] and Lemus et al. [19] have found and Canetti's proportion method as recommended by WHO [20]. Since in nitrate reduction assay the growth is detected by the colour change due to nitrate reduction as an indication, the results can be obtained much faster than visual detection of colonies. Among most of the isolates, results could be obtained within one to two weeks compared to the 4 weeks to 6 weeks time taken by the proportion method. The NRA is also easy to read due to a visible change in colour. The methods such as BACTEC 460 or Mycobacterial Growth Indicator Tube (MGIT) also give results within 7days to 10 days, but these tests are quite expensive due costly reagents and instruments. However, some genetic methods like line probe assay (Innogenetics, Belgium) [21], are fast but the cost is very high and limited to rifampicin only, also these tests can't be used in resource-poor settings. It has been reported that more than 99% of *M. tuberculosis* strains produce nitrate reductase enzyme and thus can reduce nitrate to nitrite [22].

In conclusion, β-lactam antibiotics in combination with clavulanic

acid showed a significant potential of *M. tuberculosis* growth inhibition and thus can be considered as a treatment strategy after confirming in a clinical trial. Further, when compared with the proportion method for drug susceptibility 98% to 100% agreement was observed which proved that nitrate reductase assay is rapid, inexpensive and easy to perform for early diagnosis of Mycobacterium drug resistance. Since NRA does not require expensive instrumentation and can be performed in routine laboratories with very basic facilities of LJ culture, it could be used routinely in less equipped laboratories located in the interior rural areas and villages for *M. tuberculosis* drug susceptibility. NRA may also be applied directly to microscopy positive sputa and may instantly detect drug-resistant *M. tuberculosis*.

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