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RESEARCH ARTICLE

ISOLATION AND CHARACTERIZATION OF MYCOBACTERIUM TUBERCULOSIS IN RESPIRATORY SPECIMENS

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ABSTRACT

Background & Objectives: Tuberculosis in India is one of the largest public health problems of immense consequence, with an estimate one death per minute. It is caused by bacteria *Mycobacterium tuberculosis*. Lung is most commonly affecting site, hence we aimed to isolate, and characterize *Mycobacterium tuberculosis* from respiratory specimens by conventional methods.

Materials and Methods: A total of 117 respiratory samples were included in the study. These specimens were subjected to Ziehl-Neelsen staining, culture on Lowenstein-Jensen medium, LJ with PNB and biochemical tests of Niacin, Nitrate reduction and heat stable catalase tests were performed.

Results: Out of 117 samples, 104 (88%) was smear positive, on LJ medium 104 showed growth and 3 was resistant to PNB, Niacin test was positive in 71(71%) isolates, Nitrate reduction in 79(75%) and catalase was negative in 100 (100%) of isolates.

Conclusion: The identification of Mycobacterium to the species level plays an important role in providing adequate patient management. Classical culture and biochemical tests when properly applied, detects *M.tuberculosis* in clinical samples with reasonable sensitivity.

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INTRODUCTION

Tuberculosis (TB) is a disease of great antiquity and has almost certainly cause more suffering and death than any other infection. It is contagious disease, spreads by airborne route. The disease is more common in developing countries, the problem being compounded by poverty, urbanization and overcrowding. It is one of the most important cause of increasing morbidity and mortality in India (Patil, 2013). According to the recent estimates, one third of the human population (2million people) is infected with *Mycobacterium tuberculosis* worldwide (http://www.who.int/tb/publication/ 2009/tbfactsheet_2009_one_page.pdf.Accessed on 18thoct 2009). Of these, more than half the cases occur in five South-East Asian countries. India accounts for nearly one third of the global TB burden. Nearly 500,000 people die from TB in India every year- more than 1000 every day, 1 every minute (TBC India, 2009). *M.tuberculosis* is most commonly transmitted from a patient with infectious pulmonary tuberculosis to other persons by droplet nuclei, which are aerosolized by coughing, sneezing, or speaking. Tuberculosis is classified as pulmonary and extra pulmonary. 80% of all cases of tuberculosis were limited to lungs (Braunwald, 2005).

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The clinical features of both respiratory and spinal tuberculosis were well described by Hippocrates in about 4000BC; accounts of the disease appeared in the Vedas and other ancient Hindu text, in which it was termed Rajayakshma- the king of diseases (Prasad, 2005). Tuberculosis (TB) remains one of the major causes of death from a single infectious agent worldwide. Pulmonary TB, the most important type of TB from the public health point of view, can be diagnosed by its symptoms, chest radiography, sputum smear microscopy, and by cultivation (Chan, 2000). The gold standard for TB diagnosis is the cultivation of *M. tuberculosis*. It can be performed on a variety of specimens, such as sputum and bronchial washings, and also other extra-pulmonary samples. It is much more sensitive than microscopy and it allows the recovery of the bacteria for other studies, such as drug susceptibility testing and genotyping (Palomino, 2005). Hence we aimed to isolate and characterize *Mycobacterium tuberculosis* in respiratory samples by phenotypic methods.

MATERIALS AND METHODS

This is a prospective study conducted in the Department of Microbiology, PSG Hospitals, Coimbatore during the period between June 2008 and June 2009. A total of 117 respiratory specimens were collected which includes sputum, bronchoalveolar lavage and pleural fluid.

Sample collection: About 5 to 10 ml of well coughed up early morning and spot sputum sample without salivary contamination was collected in a sterile wide mouthed, screw-capped container. BAL and pleural fluid were received in a sterile container. In case of a delay in processing immediately the samples were stored at 4°C for not more than 24hrs. Processing of all samples was done in a biosafety cabinet class IIB with universal precautions (http://www.tbcindia.org/documents.asp).

Ziehl-Neelsen Staining: A smear was made on a clean slide from thick purulent part of the sample. The smears were dried, heat fixed and stained by the Ziehl-Neelsen technique. The stained slide were examined under oil immersion, acid fast bacilli were seen as slender pink rods which was graded as per RNTCP guidelines (http://www.tbcindia.org/documents.asp).

Culture: All sputum and BAL samples were decontaminated by modified petroff's method and 1-2 loopful of the deposit was inoculated one two Lowenstein Jensen medium. Pleural fluid was centrifuged at 3000g for 15min and the sediment was used as inoculum. The bottles were incubated for 8 weeks at 37°C under aerobic atmosphere. It was then examined daily for 1 week with hand lens. The colonies of *Mycobacterium tuberculosis* were dry, rough, irregular colonies with a wrinkled surface and buff color.

Biochemical tests (Mani, 2001):

Niacin production test: 1ml of distilled water was added to the tube to extract niacin and left it for 30min, after that 0.5ml of the culture extract was pipetted into a clean screw-capped tube. To this add 0.5ml of 4% aniline in alcohol and 10% cyanogen bromide. The contents were mixed well and checked for appearance of canary yellow colour.

Nitrate reduction test: 1 loopful of culture was emulsified in 2ml of sodium nitrate. Then it was maintained at 37°C for 2hrs in water bath. After 2hrs, the tubes were brought to room temperature. And the reagents were added in the following order, 1 drop of 1:1HCL, 2drops of 0.2%sulphanilamide solution, and 2drops of 0.1% N-(1-naphthyl)-ethylene diamine di-HCL. Development of pink colour indicates positive reactions. All negative reactions were confirmed by adding a pinch of Zinc dust.

Catalase test: To the 0.5ml of 0.0067M buffer, a loopful of culture were emulsified and placed in a previously heated water bath at 68°C for 20min. The tubes were removed from water bath and cooled to room temperature. To this catalase reagent was added and observe for formation of bubble.

Susceptibility to P-Nitrobenzoic acid (PNB): Two slopes of LJ medium without drugs and one slope of LJ medium containing p-nitrobenzoic acid (PNB) at a concentration of 500µg/ml were inoculated with the neat bacterial suspension equivalent to 0.5 McFarland's standard and incubated at 37°C. Reading was taken after 28 days. *M.tuberculosis* does not grow on PNB medium.

RESULTS

Out of the 117 respiratory samples collected for isolation and characterization of *Mycobacteriumtuberculosis*, 104(88%) were sputum, 11(9%) were bronchoalveolar lavage, and 2(2%) were pleural fluid as shown in Fig 1.

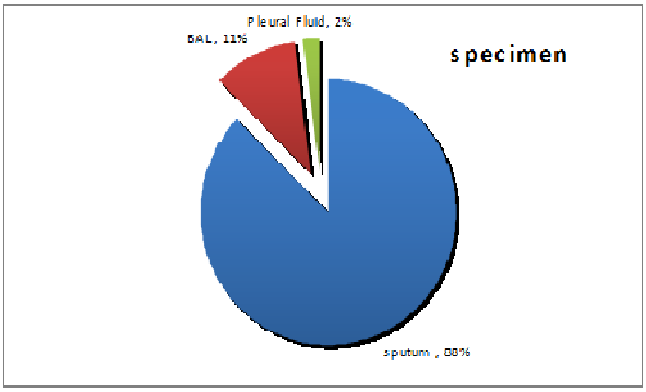

Fig. 1. Percentage of specimen distribution

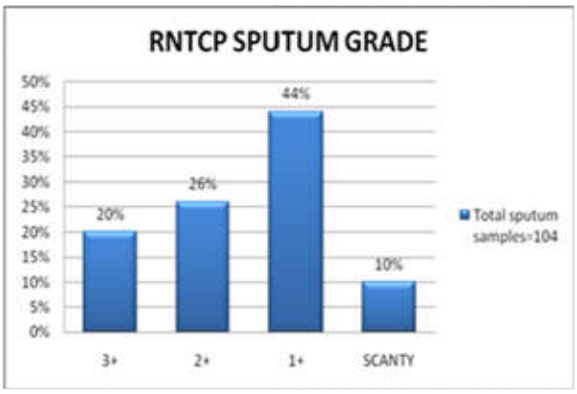

Fig. 2. Percentage of sputum samples in various RNTCP grades

The RNTCP sputum grading ranged from scanty to 3+. The distribution of various grades and the percentage of sputum samples for each grading are shown in Fig. 2. Of the 117 specimens processed, 13 had no growth and 3 samples showed growth on Lowenstein Jensen medium with p-nitro benzoic acid (PNB). Out of the 104 positive cultures, 3 which were resistant to PNB were excluded. The ratio of male is more than the females, 76% and 24% respectively giving a ratio of 3:1. The age distribution shown in Fig. 3 indicates the preponderance of tuberculosis in the fourth and fifth decade of life with the next common occurrence being in the age groups 35 – 44 and 55 – 64.

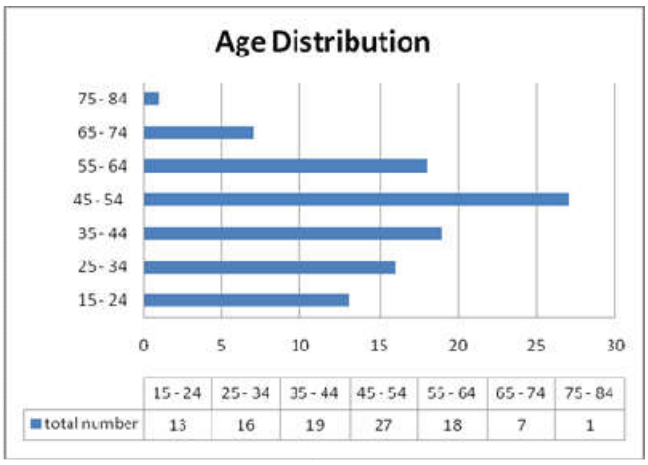

Fig. 3. Age distribution among the study group

Most of the specimens were from new cases (80%) and only (20%) comprised of old cases which included relapse and

default cases. On the basis of a panel of 3 tests the isolates were identified and characterized as belonging to *M.tuberculosis* complex. Isolates of *Mycobacterium tuberculosis* complex are positive for Niacin and Nitrate tests with negative Catalase test. Niacin test was positive in 71% isolates, Nitrate reduction in 75% and Catalase was negative in 100% of isolates as shown in Fig 4.

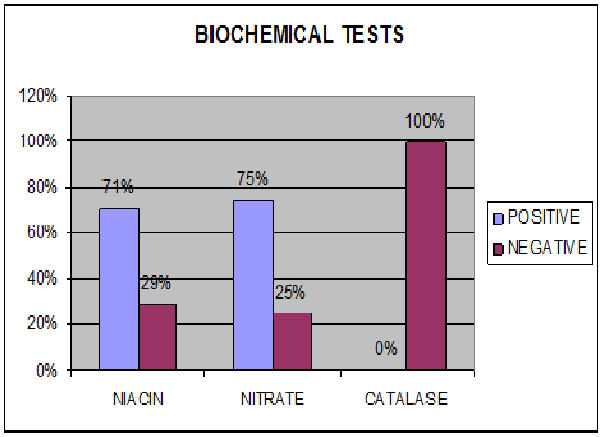

Fig. 4. Percentage of positive and negative results of the biochemical tests

DISCUSSION

India being a developing country and having unhygienic conditions provide a favorable environment for *M.tuberculosis* growth and infection. Moreover, unawareness about TB is also a source of *M.tuberculosis* transmission from infected person to healthy ones. Purpose of this study is to isolate and characterize *M.tuberculosis* from respiratory specimens by phenotypic methods. Microscopy of acid fast bacilli (AFB) is cheap and simple, detects most of the TB cases. But it is less sensitive, because a large number of bacilli must be present in a specimen for the smear to be positive. Moreover, it fails to differentiate between live and dead bacilli. In our study we identified 104 smear positive out of 117 sputum sample by Ziehl-Neelsen staining method. Similar study was conducted by Lima SSS *et al* (2008), which showed 77 smears positive out of 140 sputum samples. In our study the ratio of male and female is 3:1, similar to other studies conducted in India (Tuberculosis Research Centre, 2001 and Tuberculosis prevention Trial, 1980). In our study third and fourth decades of age groups are commonly infected. Chakaraborty *et al*. (2004), conducted a study where 25-34yr age group of people are more commonly affected. Isolation of *M.tuberculosis* by culture technique is taken as gold standard for diagnosis purpose. This bacterium grows very slowly in culture and may take several weeks for visible growth on conventional LJ medium. In our study 104 were positive for culture in LJ medium, since Non-Tuberculosis mycobacterium will also grew on LJ medium, to differentiate alternatively we used LJ with PNB, in this 3 were resistant. In a study by Varma BM *et al* (2007), 100% of the isolates were sensitive to p-nitro benzoic acid and hence was used as a reliable test along with the other biochemical tests for speciation. This correlates well with similar studies done earliar (Mondragon, 2000 and Patil,2013). The identification of *Mycobacterium* to the species level plays an important role in providing adequate patient management. Hence we characterized our isolates as *M.tuberculosis* complex by the following tests – Niacin production, nitrate reduction, Heat stable catalase at 68°C. In a study by Kothadia *et al*. (1993), 16% of the isolates were found to be Niacin negative *M.tuberculosis* whereas we encountered only 4% of such isolates. Classical culture and biochemical tests when properly applied, detect *M.tuberculosis* in clinical samples with reasonable sensitivity (Sadeghian, 2005). This has been supported by similar studies which have identified the *M.tuberculosis* by biochemical tests and molecular methods (Sadeghian, 2005; Mondragon, 2000 and Iqbal, 2003).

Conclusion

The identification of *Mycobacterium* to the species level plays an important role in providing adequate patient management. Hence we characterized our isolates as M.tuberculosis complex by the following tests – Niacin production, nitrate reduction, Heat stable catalase at 68°C. Classical culture and biochemical tests when properly applied, detects*M.tuberculosis* in clinical samples with reasonable sensitivity.

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