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Distribution and antibiotic susceptibility testing of *Mycobacterium* species present in the sputum of suspected pulmonary tuberculosis patients

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Abstract

Mycobacterium tuberculosis complex organisms are responsible for TB, which led human civilization to suffer since antiquity and still remains one of the leading causes of morbidity and mortality worldwide, even after development of effective chemotherapy and vaccination due to emergence of MDR and XDR strains. Non tuberculous mycobacteria are now considered important pathogens with high number of infections in immunocompromised as well as immunocompetent individuals.

The study was conducted during September 2010 to August 2011 at the tertiary care centre, National TB Centre, Thimi, Bhaktapur in order to classify the mycobacterial isolates obtained from sputum sample of suspected new pulmonary TB patients and to obtain the drug susceptibility profile of the isolates to the primary anti-tubercular drugs Isoniazid, Rifampicin, Streptomycin and Ethambutol.

A total of 200 sputum samples were selected for culture after screening 1500 sputum samples from suspected new pulmonary TB patients according to Bartlett pulmonary specimen culture criteria. Among them 64.50% (n=129) were culture positives, of which 87.60% belonged to *M. tuberculosis* complex and 12.40% were NTM. Among total Culture positive isolates, 19.38%, 18.60%, 18.60% and 16.28% were resistant to Isoniazid, Rifampicin, Streptomycin and Ethambutol respectively. The mycobacterial infection was found statistically significant and positively associated with family history of TB.

The study revealed that an important proportion of mycobacterial infection is caused by NTM, and there is significant drug resistance also in new mycobacterial isolates, so exact speciation and drug susceptibility testing of the isolates is necessary for the commencement of appropriate treatment to the patients.

Key words: TB, NTM

Introduction

Mycobacterium is the single genus in Mycobacteriaceae family with more than 100 species and comprises non motile, non sporing, weakly Gram positive, acid-alcohol fast, aerobic or microaerophilic, straight or slightly curved, rod shaped actinobacteria, 2-10µ in length and 0.2-0.4µ in breadth; with G+C content of DNA 61-71 mol% (except M. leprae with 54-57 mol%) and posses mycolic acid in their cell wall.[1]

Mycobacterium tuberculosis complex is a group of closely related species, comprising *M. tuberculosis, M. bovis, M. bovis* BCG, *M. africanum, M. microti*, *M. canettii*, *M. caprae* and *M. pinnipedii* which causes Tuberculosis(TB) in human and animals.[2] Other mycobacterial species that do not cause TB are called by several names like Non Tuberculous Mycobacteria(NTM), Atypical Mycobacteria, Anonymous Mycobacteria, Unclassified Mycobacteria, Unknown Mycobacteria, Tuberculoid Mycobacteria, Environmental Mycobacteria, Opportunistic Mycobacteria, Mycobacteria other than tubercle bacilli(MOTT).[1] Based on phenotypic characters, especially growth rate and pigmentation Runyon (1959) classified NTM into four Runyon groups :

Runyon group I. Photochromogens: They are slow growing NTM(i.e. require more than 7 days to appear as colony on solid media) that produce yellow to orange pigment after 24-48 hours incubation after exposure to the light source, which were not pigmented when grown in dark. Examples: *M. kansasii*, *M. marinum* etc.

Runyon group II. Scotochromogens: They are also slow growing NTM which produce yellow to orange pigment irrespective of whether they are incubated in dark or light. Examples: *M. scrofulacium*, *M. szulgai*, *M. simiae* etc.

Runyon group III. Non photochromogens: They are also slow growing NTM which do not produce pigment at all irrespective of whether they are grown up in light or dark. Examples*: M. avium, M. intracellulare*, *M. haemophilum* etc.

Runyon group IV. Rapid growers: They produce visible colonies in solid media within 7 days of incubation .They may be chromogenic like *M. vaccae*, *M. phlei*, *M. thermoresistible* or non chromogenic like *M. chelonae*, *M. fortuitum*.

Some of these NTM are involved in pulmonary mycobacteriosis while others may be involved in mycobacteriosis of other organs, while still others may be saprophytic at all or are rarely involved in disease.[3] For an immunocompromised person there may not be any non pathogenic mycobacterial species.[4]

TB is known to mankind since antiquity and has made human to suffer throughout the development upto the 21st century. It is still a major public health problem despite the discovery of its vaccination and effective chemotherapy, because of the emergence of Multi-drug resistant (MDR) and Extensively drug resistant (XDR) strains.[5] Overall one third of the world's population is currently infected with tuberculosis and 5-10% of the infected people become actively sick.[6] Twenty-two countries bear 80% of the tuberculosis burden worldwide, and 9 million people become ill with active tuberculosis and nearly 2 million people die each year.[7]

According to STC, case detection rate of TB in 2007 was 72.4% and cure rate of 86% in 2006. WHO estimates prevalence and incidence of all types of TB in Nepal as around 71000 and 48000 respectively.[8] During July 2009- July 2010 NTP registered 37732 TB cases, among which 49.5% were sputum smear positive. MDR prevalence was 2.9% and 11.7% among new and retreatment cases in 2006, and about 5% of all MDR cases registered are XDR.[9]

In vitro susceptibility testing should be performed on every first *Mycobacterium tuberculosis* isolates from patients. Susceptibility testing requires meticulous care in the preparation of medium, selection of adequate samples of colonies, standardization of the inoculums, use of appropriate controls and interpretation of results. The direct susceptibility testing method uses smear positive concentrate containing more than 50 acid fast bacilli per 100 oil immersion fields as an inoculum which provides rapid results but method is less standardized and contamination may occur. Indirect susceptibility testing method uses culture as the inoculum's source. The conventional methods used to determine drug susceptibility of *Mycobacterium tuberculosis* isolates are absolute concentration, resistance ratio, proportion and BACTEC system; whereas new methods used to test drug susceptibility use genotypic assays using PCR amplification of gene and identification of mutation, high density DNA probe assay, luciferase reporter mycobacteriophage assay.[1]

For the treatment of TB, the available anti TB drugs are Isoniazid, Rifampicin, Pyrazinamide, Streptomycin, Ethambutol, Thiacetazone, Kanamycin, Capreomycin, Para-amino salicylic acid, Ethionamide, Prothionamide and Cycloserine. The newer and experimental drugs for MDR-TB are Ciprofloxacin, Ofloxacin, Pefloxacin, Lomefloxacin, Sparfloxacin, Roxithromycin, Clarithromycin, Azithromycin and Amikacin.[10]

There are very few studies based on classification of *Mycobacterium* species isolated from sputum and the drug susceptibility pattern of isolates and more studies at tertiary care centre are necessary. The exact speciation of *Mycobacterium* is necessary for differentiation of disease as pulmonary TB or pulmonary mycobacteriosis and commencement of appropriate treatment.

Methods

The study was conducted at National Tuberculosis Centre, Thimi from September 2010 to August 2011. A total of 1500 sputum samples from suspected new PTB patients with characteristics symptoms were screened for culture of 200 sputum samples satisfying Bartlett pulmonary specimen culture criteria.[11] Three sputum samples, viz. on the spot, early morning sputum and on the spot on the following day were collected from selected 200 patients after their consent for questionnaire administration and processing of their sputum specimen. The questionnaire was filled by investigator after asking patients for their family history of TB, Smoking habit, Alcoholism habit, practice of animal husbandry and BCG vaccination questions. The sputum samples were observed by standard Ziehl-Neelsen and Fluorochrome microscopy and reported by standard criteria. The sputum samples were decontaminated and homogenized by standard N-Acetyl-L-Cysteine-Sodium Hydroxide method and cultured on paired Lowenstein Jenson media tubes. One of the two tubes was wrapped with Aluminum foil before incubation of the tubes at 37°C for 2 months before discarding the Culture negative, which was further

illuminated to light source 100W tungsten bulb placed 20 cm from the culture for 3-5 hours with cap loosened. On the basis of growth rate and pigmentation the isolates were classified to Rapid growers, Photo-chromogens, Scoto-chromogens and Non- chromogens. For each isolates biochemical tests Niacin, Nitrate reduction, Heat labile catalase and growth on PNB containing media were performed along with positive and negative controls and species identified where possible. Non chromogens were classified to *M. tuberculosis* complex and Non-chromogenic NTM on the basis of biochemical tests. The antibiotic susceptibility tests for each Culture positive isolates were performed by Standard Canetti's proportion methods to primary anti-tubercular drugs Isoniazid, Rifampicin, Streptomycin and Ethambutol.

Results

Among 200 sputum samples selected for culture by Bartlett pulmonary specimen culture criteria, 69% were positive for *Mycobacterium* by either singly or multiply by Ziehl-Neelsen microscopy, Fluorochrome microscopy or Culture. Among total positives, 90.57% were positive by Ziehl- Neelsen microscopy, 92.75% were positive by Fluorochrome microscopy and 93.47% were positive by Culture. With reference to Culture as gold standard, the sensitivity and specificity of ZN microscopy were 90.84% and 91.18% respectively and those of Fluorochrome microscopy were 94.57% and 91.54% respectively. Among Culture positive isolates, 87.60% belonged to *M. tuberculosis* complex and 12.40% belonged to NTM.

All among *M. tuberculosis* complex, were identified as *M. tuberculosis* isolates and among NTM isolates, 81.25% were Nonphotochromogens, 12.5% were Scotochromogens and 6.25% (n=1) was Rapid grower which was identified as *M. vaccae*; photochromogens were not isolated at all.

Among *M. tuberculosis* complex only 7.96% of *M. tuberculosis* isolates were resistant to anti-tubercular drugs, all MDR, of which 11.11% was resistant to INH and RMP, 22.22% were resistant to INH, RMP and STR, and remaining 66.67% were resistant to all four tested anti-tubercular drugs INH, RMP, STR and EMB. Among 16 NTM isolates, 6.25% isolate was resistant to INH and STR only, but all other 93.75% isolates were resistant to all four tested anti-tubercular drugs, INH, RMP, STR and EMB.

The mycobacterial infection in patients was statistically significant to Family history of TB (χ^2 9.44, $p<0.05$) and practice of animal husbandry (χ^2 4.23, $p<0.05$) but the association of attribute was positive for family history of TB (Q=0.46) and negative for practice of animal husbandry (Q=-0.3). The mycobacterial infection in patients was statistically insignificant ($p>0.05$) to Smoking, Alcoholism habit and BCG vaccination.

Discussion

Sputum is the material coughed up from the lower respiratory tract and expectorated from the mouth, it contains mucous, cellular debris, microorganisms and possibly blood or pus; the amount, colour and constituents of the mucous are important in the diagnosis of many illnesses, including PTB, pneumonia and lung cancer.[12] The criteria developed by Bartlett rejected 17% of specimens for culture but missed fewer potential pathogens so economical for expensive culture of sputum.[13]

In the present study, the number of patient positive for *Mycobacterium* by either culture or ZN microscopy or fluorescent microscopy were 138 among which 71.01% were male and 28.99% were female which is similar to 69% male and 31% female among 15,468 new smear positive cases recorded during July2008-July2009 affecting mostly 15-45 year age group, i.e. productive age group, so greatly affecting the economic and social status of the country.[9] Male population is slightly less than female population as seen in Census 2011 in Nepal but the gender differentiation in TB may be due to exposure of male to external environment or males visiting health centre independently for disease diagnosis but low detection of female TB cases remains a troubling public health issue demanding urgent focused study.[14]

The comparison of ZN staining to the culture showed the sensitivity 90.84% and specificity 91.18%, which showed that ZN staining, is quite efficient in the diagnosis of mycobacterial infection in the lung, which is economically viable in low resource setting countries. The comparison of fluorescence staining to the culture, showed the sensitivity 94.57% and specificity 91.54% which is quite efficient in preliminary disease diagnosis and provides result in few hours as compared to culture taking weeks or up to months to provide result, although culture is useful for the performance of bio-chemical tests to identify the species for definitive disease diagnosis and to provide colonies for the drug susceptibility tests. The lower magnification of 200-250× allows examiner to observe lesser fields than by ZN microscopy for the diagnosis which made fluorescent microscopy faster and quite useful in those laboratories where many slides are to be observed in a day. Respiratory specimen yields higher smear positivity rates and if more than one specimen is submitted to the laboratory, up to 96% of patients with PTB may be detected by acid fast stains and smear positivity is correlated with the number of colonies recovered in culture.[15]

Among 129 Culture positive cases, 87.60% (n=113) person had TB and 12.40% (n=16) had pulmonary mycobacteriosis. In some laboratories NTM are more commonly isolated from respiratory secretions than *M. tuberculosis* and pulmonary

mycobacteriosis are probably under-diagnosed. Pulmonary mycobacteriosis caused by MAC predominate and account for 48-70% of all NTM infections. Significant geographic variability exists both in the prevalence and species responsible for NTM disease.[3]

Overall 1/3rd of the world's population is currently infected with TB and 5-10% of infected people become actively sick (WHO, 2011). Among 113 culture isolates of *M. tuberculosis* complex responsible for PTB, only 7.96% (n=9) of *M. tuberculosis* isolates were found to be resistant and all resistant *M. tuberculosis* strains were MDR strains resistant to at least INH and RMP. The MDR strains prevalence of TB patients is continually increasing and the MDR prevalence of TB patients never previously treated for TB was 2.9% and among retreatment cases was 11.7% in 2006.[9]

Among 16 NTM isolates, 93.75% of total NTM strains isolated were resistant to all four drugs and 6.25% (n=1) was resistant to INH and STR only. Strains of MAC are intrinsically resistant to anti-tuberculosis drugs and many other antimicrobial agents owing to failure of these drugs to penetrate the lipid rich cell wall. Almost all strains of rapidly growing mycobacteria are resistant to anti-tubercular drugs and antimicrobial agent used for treatment depends on identification of the isolate and results of drug susceptibility studies.[16]

There may be various risk factors for TB because the number of causal chains is essentially infinite because causation can be expressed on basis of physiological, genetic and behavioural factors; so on the basis of life-cycle of *M. tuberculosis* risk factors may be categorized during infection, progression to disease and adverse outcome of disease.[17] Risk factors for infection include spatial proximity to infectious TB patient in household setting or due to work in hospital. Progression to disease may be facilitated by co-morbidities, such as HIV/AIDS, Diabetes or silicosis, as well as by malnutrition.[18] Smoking and alcoholism may both be associated with an increased risk for TB through an increased iron content in broncho-alveolar macrophages leading to reduced host defence towards intracellular micro-organisms.[19,20] Adverse outcome is directly or indirectly associated with alcoholism, intravenous drug use, homelessness and malnutrition. Poverty is itself related to a number of above risk factors.[18]

In present study, the presence of mycobacterial infection in patients was studied with risk factors family history of TB, smoking, alcoholism, animal husbandry and BCG vaccination. Among these the association between mycobacterial infection and family history of TB and animal husbandry were statistically significant but mycobacterial infection was only positively associated with family history of TB; it depicts the infectious nature of family member with TB and presence of contacts with TB cases and duration of contacts are important for transmission of disease. The negative association of mycobacterial infection and animal husbandry may be due to influence of extraneous variables like more practice of animal husbandry in sub-urban and rural areas where there is comparatively less population density, less air

pollution which may be important for transmission and development of lung diseases.

Conclusion and Recommendations

Hence, the mycobacterial isolates obtained in culture from sputum of suspected new pulmonary TB patients were classified and their drug susceptibility patterns obtained. Following recommendations are made on basis of this study:

- *Mycobacterium* species should be identified to the species level for the treatment of mycobacterial diseases.
- Drug susceptibility testing of each new *Mycobacterium* isolates is recommended.
- The family contact of infectious TB cases should be considered as high risk individuals.
- The choice of antibiotics used for treatment should be determined by drug susceptibility testing studies.

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