SECONDARY BACTERIAL INFECTION IN CLINICALLY SUSPECTED CASES OF PULMONARY TUBERCULOSIS AND THEIR ANTIBIOTIC SENSITIVITY PATTERN

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ABSTRACT

Secondary bacterial infection has become one of the most important complications in patients suffering with pulmonary tuberculosis (PTB). The suppression of immunity, which can occur due to T-lymphocyte deficiency during active PTB disease, could be the main reason for the bacterial superadded infections. This aimed to evaluate tuberculosis (TB) status and secondary bacterial infections among them. A total of 400 sputum samples were collected and examined using Ziehl-Neelsen staining method as per revised national tuberculosis control program guidelines and GeneXpert following the WHO guidelines. Those sputum samples were also processed for routine bacterial culture and bacteria were identified by conventional techniques. Antimicrobial sensitivity testing was performed on Mueller-Hinton agar plates with commercially available antibiotic discs using Kirby-Bauer disc diffusion techniques and interpreted as per the guidelines of Clinical and Laboratory Standards Institute (CLSI). A total of 44 (11.0%) samples out of 400 yielded tuberculosis by GeneXpert assay and maximum positivity was noted among the age group 46-60 years (20.4%). The present study showed GeneXpert positivity for the MTB detected rate remained to be 11.0 % (44/400) detected as against smear positivity in only 7.5% (30/400) and this difference was found to be statistically significant ($p<0.001$). Overall, 220 (55.0%) secondary bacterial pathogens were isolated. Gram negative bacterial infection was most common. Of them, *Klebsiella pneumoniae* (33.63%; 74/220) was the commonest organism followed by *Acinetobacter* spp. (30%;66/220) and *Pseudomonas aeruginosa* (18%; 40/220). *Streptococcus pneumoniae* (6.36%; 14/220) was the commonest among the gram-positive bacteria. Majority (54.0%) of *Klebsiella* spp. showed resistance to Ciprofloxacin, while 29.7% Imipenem, 32.4% to Gentamicin, 35.13% to Piperacillin/Tazobactum, 37.8% to Amikacin. However, (70.0%) of *Acinetobacter* spp. showed resistance to Ciprofloxacin, while 75.7% were resistant to Ceftazimine, 54.5% Imipenem, 48.4% to Gentamicin, 31.0% to Amikacin. Interestingly, 44 of these 400 (11.0%) were found to positive be MTB by GeneXpert test and 220/400 (55.0%) bacterial infection and coinfection 22/44 (50.0%) among clinically suspected PTB cases. Tuberculosis remains a global threat despite effort to eradicate the disease and PTB co-infection with secondary bacterial infection may complicate the infection and treatment.

KEYWORDS

Bacterial infections, tuberculosis, ZN stain, GeneXpert

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INTRODUCTION

Pulmonary tuberculosis (PTB) is placed amongst the top 10 causes of death worldwide.[1] Suspected tuberculosis is defined as any person when presents with symptoms or signs suggestive of PTB.[2] The most common symptoms of PTB is a productive cough for more than 2 weeks, accompanied by other respiratory symptoms (shortness of breath, chest pains, hemoptysis) and/or constitutional symptoms (loss of appetite, weight loss, fever, night sweats, and fatigue).[2] More than 95.0% of PTB cases have been reported from developing countries, particularly from Asia, Africa, the Middle East and Latin America having limited diagnostic and therapeutic facilities.[3,4] There are many risk factors for acquiring TB such as family history of close contact with TB patients, social status, age, poverty, male gender, HIV infection, smoking and homelessness.[5]

Upper respiratory tract infections (URTIs) are commonly caused by viruses rather than bacteria and fungi but lower respiratory tract infections (LRTIs) are more commonly caused by bacteria and less often by fungi and viruses.[6] These diseases associated with 7 million deaths annually.[7] Several studies carried out world-wide[8,9] report that the potent pathogens of lower respiratory tract infections are *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Klebsiella pneumoniae*, *Acinetobacter* spp., *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, *Moraxella* spp. etc.

Secondary bacterial infection has become the most important complications in patients suffering from PTB as there is inhibition of human defense mechanism during the course of active tuberculosis and because of altered immune status of PTB patients.[10] Moreover, the alveolar linings material of patients with active pulmonary tuberculosis has less bactericidal activity.[10] The co-infection of PTB with other LRTI has been documented in the literature, particularly in areas where PTB is prevalent.[9,11]

The suppression of immunity, which can occur due to T-lymphocyte deficiency during active PTB, could be the main predisposition for the coinfection.[10] Hormonal alterations, such as suppressed pituitary function, increased adrenal and pancreas functional activity, higher cortisol levels, and altered thyroid function, occur during the early stages of PTB and may lower immune competency.[10] Emergence of bacterial coinfection along with the development of antimicrobial resistance complicates the TB-treatment process.[10] Hence, this study was designed to explore the bacterial spectrum of secondary infections among patients suspected with PTB and to determine the antibiotic susceptibility pattern of the isolates among patients visiting Manipal Teaching Hospital, Pokhara, Kaski.

MATERIALS AND METHODS

This was an analytical observation study conducted between August 2024 - March 2025 in the Department of Microbiology, Manipal Teaching Hospital (MTH), Pokhara, Nepal after obtaining approval from the Institutional Research Committee (IRC) of Manipal College of Medical Science (Ref. No.: MEMG/IRC/555).

A total of 400 sputum samples were collected by consecutive sampling technique. The sample size was determined using the formula sample size n = z^2P (1-P) \ d^2N = z^2P (1-P) \ d^2, where n = sample size, z = level of confidence (1.96), P = expected prevalence, here in this study P = 47.0% ([12]) d = precision (0.05) and minimum sample size obtained this formula was 400. Patients clinically suspected to have PTB with symptoms of cough for more than 2 weeks accompanied by shortness of breath, chest pains, hemoptysis and/or constitutional symptoms of loss of appetite, weight loss, fever, night sweats, and fatigue[2] visiting MTH were included in this study. All the sputum samples from the clinically suspected PTB cases brought to microbiology laboratory for ZN staining, GeneXpert and for bacteriological culture were included, whereas, samples from patients undergoing any antibiotic treatment were excluded from study.

Morning sputum specimens were collected from all patients after proper instructions so to get ideal sample in a falcon tube of 50 ml capacity. At least five milliliters of sputum were collected from each patient. In case of in-patients who were unable to provide samples, sputum production was induced by nebulization with hypertonic saline.[13] All samples were sent to the Department of Microbiology, MTH with no delay.

From the thick and purulent part of sputum, a smear was prepared on a clean grease free glass slide using a disposable wooden applicator stick. The smear was air dried, heat fixed and stained with Z-N staining method as per the revised national tuberculosis control program (RNTCP) guidelines.[14] Acid fast bacilli were seen as bright red/pink rods against blue background.

GeneXpert assay was done in accordance with the WHO recommended guidelines.[15] About 3 ml of the specimen was mixed with twice its volume of sample reagent. The mixture was then vortexed and incubated at room temperature for 10 minutes. Thereafter, it was again vortexed and incubated for another five minutes. About 2 ml of this processed sample was then added to GeneXpert cartridge which was then loaded into the machine. The results were interpreted by the GeneXpert system based on fluorescent symbols which was displayed on the system monitor after about two hours. The data were collected, entered and analyzed using SPSS-18. Categorical variables were calculated as percentages. Chi-square test was employed for statistically comparisons. P values <0.05 was considered as statistically significant.

For bacterial isolation, all the sputum samples were inoculated in blood agar and chocolate agar incubated at 37°C. All bacteria were identified by conventional techniques.[16,17] Antimicrobial sensitivity testing was performed on Mueller-Hinton agar plates with commercially available antibiotic discs using Kirby-Bauer disc diffusion techniques and interpreted as per the guidelines of Clinical and Laboratory Standards Institute (CLSI).[18] The antibiotics (Himedia, Mumbai, India) and their concentration per disc (µg) were: Ampicillin (10µg), Piperacillin/ Tazobactam (100/10µg), Ciprofloxacin (5µg), Amikacin (30µg), Imipenem (10µg), Gentamicin (10µg), Tigecycline (15µg), Ceftriaxone (30µg), Ceftazidime (30µg) for Gram negative bacteria and Erythromycin (15µg), Amikacin (30µg), Gentamicin (10µg), Ciprofloxacin (5µg), and Clindamycin (2µg), Vancomycin (30µg), Teicoplanin (30µg) for Gram positive bacteria. The European Centre for Disease Prevention and Control (ECDC) criteria were taken into consideration for categorizing an isolate as multidrug resistant.[19]

RESULTS

This study aimed to evaluate TB status and coinfection of TB with pulmonary secondary infections in patients visiting Manipal Teaching Hospital, Pokhara. Table 1 depicts the number of male visiting to the hospital is more (69.5%) as compared to females (30.5%).

Distribution of patient according to their age groups has been depicted vide Table 2. As shown, individuals belonging to this age group of 46–60 or above were more frequently infected as compared to the individuals in other group categories.

Table 3 depicts the total positivity and the positivity rates of PTB as tested by GeneXpert assay. A total of 44 (11.0%) samples out of 400 yielded *M. tuberculosis* by GeneXpert assay. Maximum positivity was noted among subjects belonging to of age group 46-60 years (20.4%), emphasizing that higher age group could be one of the risk factors for pulmonary secondary infection. Contrary to this, Z-N smear positivity was found in 30 (7.5%) samples only as shown in Table 4. This table also depicts the relationship between GeneXpert positivity and ZN smear positivity. Out of 44 GeneXpert positive samples, 30 had yielded high and medium bacillary density and 14 yielded low, very low and trace bacillary density. Whereas all 30 specimens showing high and medium grade positing in GeneXpert were also positive by staining, those yielding very low or trace MTB by GeneXpert were negative by staining. The above observations, undoubtedly signified the superiority of GeneXpert assay over microscopy. GeneXpert could detect MTB from number of samples that were negative by the smear examination. (low 8, very low 1, trace 4). None of the samples showed rifampicin resistance (0.0%).

Table 1: Distribution of patients according to gender

Gender	Number	Percentage
Male	278	69.5
Female	122	30.5
Total (400)	**400**	**100**

Table 2: Distribution of patients according to age group

Age Group	Number	Percentage
1-15yr	16	4
16-30 yr	16	4
31-45 yr	**22**	**5.5**
46-60 yr	**98**	**24.5**
More than 60yr	**248**	**62.0**
Total	**400**	**100**

Table 3: Distribution of tuberculosis cases from clinically suspected TB according to age group

Age Group	Tuberculosis		Total
	MTB Not detected	MTB detected	
1-15 year	16	0	16
16-30 year	12	4	16
31-45 year	20	2	22
46-60 year	78	20 (20.4%)	98
More than 60 year	230	18	248
Total	**356**	44 **(11%)**	**400**

Table 4: Density of M tuberculosis (MTB) detected by GeneXpert Ultra as compared to Z-N smear positivity

Density of MTB in GeneXpert (No of samples positive)	ZN Staining		
	Acid Fast Bacilli not seen	Acid Fast Bacilli seen	Total
High (16)	0	16	16
Medium (14)	0	14	14
Low (8)	8	0	8
Very Low (2)	2	0	2
MTB Not detected	356	0	356
Trace (4)	4	0	4
Total	370	30	400

(P value significant< 0.001)

Table 5: Correlation of GeneXpert and ZN staining positivity

GeneXpert	Z-N positive (%)	Z-N negative (%)	Total	
Positive (44)	30 (68.18)	14 (31.82)	44	*p <0.001
Negative (356)	0 (0.0)	356 (100)	356	
Total (400)	**30 (7.5)**	**370 (92.5)**	**400**	

Out of a total of 44 Gene Xpert positive samples, 30 i.e. 68.18% were positive by staining. As compared to this, of the 356 Gene Xpert negative samples, none yielded positive results by staining. This difference was found to be statistically significant (p <0.001; Table 5). Interestingly, 22 of those 44 GeneXpert positive samples had yielded bacterial co-infection, reflecting all almost 50.0% of other superimposed bacterial infections among TB patients.

Of the total 400 samples, secondary bacterial infections were detected in 220 (55.0%) (Table 6). As shown in Table 6, Gram negative bacterial infection was the predominant. *K. pneumoniae* (33.63%;74/220) was the commonest organism followed by *Acinetobacter* spp. (30%; 66/220) and *P. aeruginosa* (18.18%; 40/220). *Str. pneumoniae* (6.36%; 28/220) was the commonest among the gram-positive bacteria.

Table 6: Distribution of pathogens other than MTB Isolated from same sputum samples

Microorganism	n of secondary pathogens (%)
Gram negative bacteria	
Acinetobacter	66 (30.0)
Klebsiella pneumoniae	74 (33.63)
Pseudomonas aeruginosa	40 (18.0)
Escherichia coli	8
Proteus	6
Gram positive bacteria	
Staphylococcus aureus	10 (4.54)
Alpha hemolytic streptococci	14 (6.36)
Enterococcus	2
No Growth	180
Total	**400**

DISCUSSION

PTB is a chronic condition that weakens the immune system, causing structural changes in the lungs (bronchiectasis, cicatrization, and scarring) leading to abnormal functioning and creating an opportunity for secondary bacterial infections.[20,21] However, co-existence of PTB and bacterial infections in immunocompetent patients is rarely documented when *M. tuberculosis* co-infects with other bacterial pathogens, such as *Klebsiella* and *Pseudomonas*, it can exacerbate the patient's clinical condition, the severity of TB and elevate the risk of complications.[22] Coinfections can result in more extensive lung damage and necessitate longer treatment durations.[24] The epidemiological study and best diagnostic approaches for co-infection are still under investigation.[23]

In regions with high TB prevalence, where TB treatment often relies on presumptive diagnosis as the data on the co-infection of TB with other pathogens remain scarce.[9,12] Due to the lack of studies on co-infection, in areas of high burden of TB, this study could provide valuable insights into the actual prevalence of bacterial co-infections among known PTB cases. Treating patients with co-infection becomes challenging due to the distinct treatment approaches required for each condition.[9]

In this present study, of all the patients with clinically suspected PTB, males happened to be more in number than females. This could be attributed to the fact that the higher exposure of males and the productive age group to external environment and surroundings. The additional reasons may also be due to increased incidence of smoking and also because of greater access to healthcare facilities in developing countries.[24] This observation was in agreement with the reports of WHO indicating male gender as a predisposing factor for tuberculosis infection.[3] With regard to the age, age group of 46–60 or above were under more threats of being infected as people in this age group had declined level of immunity and their exposure to the environment was high.[25]

A total of 11.0% samples yielded *M. tuberculosis* by GeneXpert assay. Maximum positivity (20.4%) was noted among subjects belonging to of age group 46-60 years. This is the age when there is changes in the physiologic functions including less production of microbiocidal peptide as well as lysozyme take place.[26] Therefore, the age group is also one of the risk factors for pulmonary secondary bacterial infection mainly due to diminishing immunity.

In the present study, GeneXpert positivity for the MTB remained to be 11.0% against the smear positivity in only 7.5% of the samples included. Hence, it is needless to emphasize that GeneXpert assay being a molecular tool overcomes the limitations of smear microscopy which mainly depends upon factors like technical expertise in microscopy, and bacterial load in a particular sample. This study highlighted that samples reported negative in smear examination could be detected by GeneXpert assay. Similar observation was made by Umair *et al*[27] (they reported that 30 samples positive among 50 GeneXpert positive samples). Notwithstanding the low positivity of ZN smear, this technique cannot be totally ignored. Besides being a rapid and user-friendly tool, its results were found to be in good agreement with the density of mycobacterial yield detected by GeneXpert.

Overall, secondary infections by pathogens were 55.0% (out of total 400 specimens). Gram negative bacteria were most common (18.0%) compared to gram positive bacteria (11.81%). Among the Gram-negative bacteria, *K. pneumoniae* (33.63%) was the commonest organism followed by *Acinetobacter* spp. (30.0%) and *P. aeruginosa* (18.18%). *Str. pneumoniae* (6.36%) was the commonest among the gram-positive bacteria. Attia *et al*[22] reported similar

findings, where *K. pneumoniae* and *P. aeruginosa* were the predominant causes of respiratory infections, both in cases with and without TB co-infection.[22] Bir *et al*[28] in their study showed that among the bacterial pathogens isolated, *P. aeruginosa* was the most common, accounting for 36.84%, followed by *Acinetobacter baumannii* (31.57%), *K. pneumoniae* (26.31%), and *Stenotrophomonas maltophilia* (5.28%). The higher relative abundance of gram-negative bacteria may be attributed to antibiotic pre-treatment within our population. The presence of gram-negative bacteria cultured from sputum could also indicate colonization of abnormal lung architecture resulted from TB and lowered immunity due to TB.

Regmi *et al*[12] observed that *P. aeruginosa* as most predominant bacterial isolate recovered in 9.33% of the total sample. A similar result was found in in Pakistan, where the prevalence of *P. aeruginosa* was 11.96%.[29] The frequent incidence of *P. aeruginosa* in the sputum may be owing to their ability to colonize a wide range of ecological niches, such as air polluting agents, animal hosts and humans.[30]

Majority (54.0%) of *Klebsiella* spp. showed resistance to Ciprofloxacin, while 29.7% to Imipenem, 32.4% to Gentamicin, 35.13% to Piperacillin Tazobactam, 37.8% to Amikacin. However, (70.0%) of *Acinetobacter* spp. showed resistance to Ciprofloxacin, while 75.7 % were resistant to Ceftazidime, 54.5% to Imipenem, 48.4% to Gentamicin, 31.0% to Amikacin. Overall, 110 (55.0%) bacterial pathogens were isolated. Out of 33, a total of 14 *Acinetobacter* spp. (42.42%) was Multidrug resistant. All the isolates of *Acinetobacter* were susceptible towards Colistin and Polymyxin B. Out of 37 *Klebsiella*, 21.0% were multidrug resistant. All the *Klebsiella* isolates were susceptible towards Colistin, Polymyxin B and Tigecycline.

Regmi *et al*[12] documented 55.56% MDR isolates which was higher than the study observed out in Kathmandu (47.57%) by Pokhrel *et al*.[31] Interestingly, a total of 400 clinical samples, 44 were found to be MTB detected by GeneXpert. And secondary bacterial coinfection was observed in 50.0% of the diagnosed TB cases which is (50.0%; 22/44). In the study done by Attia et al., reported that 33% of TB patients were found to have bacterial co-infection.[22] Similarly, Liu *et al*,[32] observed that 31.4% of patients had both TB and bacterial co-infection. It may be assumed that some of the devastating effects of TB on infected persons might result from the synergistic effects of both the organisms.

The structural changes in the lung parenchyma, including bronchiectasis, cicatrization, and scarring, can impact normal pulmonary function.[22] When *M. tuberculosis* co-infects with other bacterial pathogens, such as *Klebsiella* and *Pseudomonas*, it can exacerbate the severity of TB and elevate the risk of complications.[22] Co-infections can result in more extensive lung damage and necessitate longer treatment durations.[2] At the same time, it is also true that structural lung damage associated with PTB coupled with the prolonged use of anti-TB drugs, some of which also have activity against common bacterial pathogens of community-acquired pneumonia, may alter the profile of bacteria that colonizes the respiratory tract.[33] Therefore, it is imperative to have a clear understanding of the profile of bacterial aetiology of secondary pneumonia in patients with active PTB.[33]

There is evidence indicating that the long-term use of antibiotics might be related to alterations in the resident oral microbiota and to a possible increase in the occurrence of opportunistic microorganisms, such as enteric bacilli, *Pseudomonas* spp. and *Staphylococcus* spp.[34] Feldman *et al*[35] showed chronic chest infection and the use of antibiotics are risk factors for colonization with *K. pneumoniae*. Therefore, the use of multiple antimicrobial agents in the treatment of PTB could have explained the pattern of the microbial pathogens observed as the causative agents of secondary bacterial pneumonia among patients with active PTB.[33]

Despite global efforts to eradicate, TB remains as an infectious disease has remained a global threat. Co-infection with secondary bacterial infections in clinically suspected tuberculosis had been recognized for its wide range of clinical spectrum and chronicity.[36] The findings of present study showed that high prevalence of bacterial co-infection in pulmonary TB patients. *K. pneumoniae* were the commonest organism followed by *Acinetobacter* spp. and *P. aeruginosa*. When *M. tuberculosis* co-infects with other bacterial pathogens, such as *Klebsiella* and *Pseudomonas*, it can exacerbate the patient's clinical condition, the severity of TB and elevate the risk of complications.[23] Co-infections can result in more extensive lung damage and necessitate longer treatment durations. Customizing treatment regimens based on individual patient profiles becomes

crucial for achieving favorable outcomes in patients with co-infection due to MTB and other bacteria. Further studies on the epidemiology of such infections and improved diagnostic approaches for co-infections will help in better patient management.

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