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Original Research Paper

Medical Microbiology

BACTERIAL PROFILING AND MOLECULAR CHARACTERIZATION OF BRONCHOALVEOLAR LAVAGE FROM CHRONIC RESPIRATORY DISEASES PATIENTS ATTENDING A TERTIARY CARE CENTER IN THANJAVUR.

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ABSTRACT Chronic respiratory infections are a major public health problem in India as well as worldwide. Pulmonary infections are attributed to be the most prevalent cause of exacerbations in this group of diseases. The application of Broncho alveolar lavage (BAL) as a specimen for diagnosis has improved the sensitivity and specificity of detecting pulmonary infections. BAL fluid from Chronic respiratory diseases cases were collected prospectively and analyzed for bacterial isolates. Antimicrobial susceptibility testing was done for bacterial isolates. Molecular characterization was done for the commonest isolated organism. In a sample population of 100 BAL samples, 35samples showed positive growth. Out of which 25 were bacterial isolates. *Kelbsiella pneumonia* constituted 28% and was the commonest bacterial pathogen. High antimicrobial resistant was noted in *Acinetobacter baumanii*. Gram negative bacteria exhibited more antimicrobial resistance than Gram positive organisms.100% sensitivity was observed to Linezolid and Vancomycin. Pulmonary infections were more prevalent in COPD among chronic respiratory diseases. Bronchoalveolar lavage despite being an invasive sample proves to be a reliable specimen for quantitative diagnostic procedures due to increase the sensitivity and. Gram negative bacteria are emerging as primary pathogens with high antibiotic resistance and every healthcare set up requires an updated antibiogram for the judicious use of antibiotics.

KEYWORDS : Bronchoalveolar lavage, Antibiotic sensitivity, Klebsiella, Chronic respiratory diseases

INTRODUCTION:

Chronic respiratory disease affects the airways and other structures of lungs. This group consists of diseases like COPD, emphysema, bronchitis, asthma, chronic pleural diseases, pneumoconiosis, pulmonary eosinophilia, sarcoidosis, sleep apnea syndrome and pulmonary heart diseases. Other conditions like cystic fibrosis, pulmonary fibrosis and occupational lung diseases are included in this group [1]. Usual presentations of these diseases were, cough, pain in throat or chest, abnormalities of breathing, bleeding of respiratory passages. Signs involving respiratory and circulatory system were asphyxia, pleurisy, productive sputum and cardio respiratory arrest. Chronic respiratory diseases contribute four million deaths per year worldwide, contributing to 5% of deaths annually [2]. In India, chronic respiratory diseases were found to be the reason for 7% of deaths, and DALYs lost were 3% [3]. Exacerbations seen in these diseases are associated with greater and irreversible decline in lung function, significant mortality and morbidity [4]. Almost 75 to 80% of infections seen in these diseases were bacterial and viral pathogens. Frequently associated bacteria involved in exacerbations include *Haemophilus influenzae*, *Streptococcus pneumonia* and *Moraxella catarrhalis*[3] Infections are most frequent cause of exacerbations [4]. Making an earlier diagnosis and usage of appropriate antimicrobials is a must for management of these patients. Diagnosis by way of sputum culture is seen in less than 50% of patients with pneumonia. Lower respiratory tract infections produce between 5 to 10 % of all deaths reported to the CDC via Mortality Reporting System as per WHO [5].

Clinically visible chronic respiratory diseases are the ones usually diagnosed rather than the hidden entities. Bacterial colonization of the distal airways, though poorly understood, can worsen the underlying disease. The constant presence of microorganisms in the distal airways can lead to severe progression of the conditions. We need to know the type of colonizing microbial agents, which can help in devising antibiotic protocols for treating these conditions affecting the lower respiratory tract. This has envisaged the importance of acquiring the quantitative invasive procedure like BAL for identifying the pathogen and aiding in their diagnosis and management. With the arrival of bronchoscopy and quantitative invasive techniques like Bronchoalveolar lavage showed a marked improvement in the sensitivity and specificity of these diagnostic techniques in detection of pulmonary pathogens [6]. BAL has the dual advantage of being the most appropriate one for most of the microbiological procedures, and usually has adequate volume to perform multiple tests. The procedure has become easily accessible to the community and is available at an affordable cost. In this study to evaluate the patients of chronic respiratory diseases who underwent BAL, which was processed in the central diagnostic laboratory, department of microbiology and the results obtained were analyzed.

MATERIALS AND METHODS:

Study population:

This study included 100 patients (men and women above 18 years) who underwent bronchoscopy. This study was conducted over a period of one year from May 2015 to April 2016. The ethical committee of this institution approved the study and informed consent was obtained from all patients undergoing the study.

Examination of Patients.

In the Thoracic Medicine ward, patients with Chronic respiratory diseases where asked for history of presenting complaints like fever, cough and production of sputum. Duration of illness. History of any cardiac problems.

Specimen Collection.

Patients were instructed to stop smoking and fast for a minimum of 12 hrs before sample collection. Bronchoscopy with BAL was performed according to standardized procedures designed to minimize oral contamination. BAL was performed by sequentially instilling and then withdrawing 50 ml aliquots of sterile normal saline. Bronchoalveolar lavage fluid specimens collected under aseptic precautions was immediately transported to the laboratory for Direct Microscopy and bacterial culture processing.

Bacterial culture

For bacterial culture uncentrifuged samples were used. The sample was inoculated for quantitative bacterial culture using

standard laboratory techniques on Blood agar, Chocolate agar and Mac conkey agar using a sterile 4mm nichrome loop (0.01 ml) and incubated at 37°C for 72 hours. Colony count of >105 colony forming units per milliliter of BAL fluid was taken as the cutoff for bacterial infection.

Identification of Bacteria

The culture plates were examined for the presence of bacterial colony and the growth was identified with Gram's staining, Catalase test, oxidase test, hanging drop test, Biochemical reaction and carbohydrate fermentation.

Antimicrobial susceptibility testing

Antibiotic susceptibility testing was done for bacterial isolates by Kirby- Bauer's disc diffusion method on Muller-hinton agar plate. Antimicrobial susceptibility testing was performed by agar disc diffusion method according to Clinical and Laboratory Standards Institute (CLSI) guidelines.

Preparation of turbidity standard

Commonly used is the McFarland 0.5 standard. This contains 99.5 ml of 1 % sulphuric acid and 0.5 ml of 1.175 % barium chloride. The solution is poured in the tube, tightly sealed and stored at room temperature at a dark place. This is comparable with bacterial suspension of 1.5 X 108 colony forming unit/ml.

Preparation of Inoculum

For preparation of the Inoculum, 3 to 5 colonies were lifted and inoculated in 5 ml of peptone water and incubated at 37°C for 2 to 6 hours in order to obtain 0.5 McFarland standard which corresponds to 150 million organism per ml. If more turbidity was noticed, peptone water was added to adjust it to 0.5 McFarland's standard.

Inoculation of MHA plates

A sterile cotton swab was dipped and rotated several times within 15 minutes of adjusting the turbidity of the inoculums. To be remove excess fluid the swab was pressed firmly on the inner side of the wall above the level of the fluid. By streaking method the dry surface of the Muller-Hinton agar plate was swabbed. This method was repeated 2 or more times by rotating the plate to 60°to cover the entire surface of the plate. Finally the rim of the agar plate was swabbed. The plate was closed and allowed to wait for 3 t0 5 minutes for excess moisture to be absorbed before applying drug impregnated discs.

Application of discs to inoculated agar plate

A set of predetermined battery of antimicrobial discs were tested on all isolates. The whole disc was placed on the agar surface and pressed to make sure the entire surface of the disc comes in contact with the agar. Even distributions of the discs were making to ensure 25 mm distance from centre to centre of discs. This was followed by incubation at 37°C for 16 – 18 hrs.

Reading and interpretation of results

Each plates were examined after 16 – 18 hours for satisfactory streaking with uniform circular zones of and confluent lawn of growth. The diameters of the zones of complete inhibition including the diameter of the discs were measured. The zone was measured to the nearest millimeter using a ruler which was held on the back of the agar plate. The zone of inhibition size was interpreted by referring to CLSI guidelines and reported the organism as resistant, intermediate or susceptible.

Molecular identification

All the isolates were grown on Muller Hinton Agar and were incubated for 48 hours at 37°C under aerobic conditions and the bacterial colonies were then used for DNA extraction. Molecular characterization was done for the commonest isolated organism by Polymerase Chain Reaction.

Statistical Analysis:

Data collected will be analyzed using descriptive statistical methods by computing Percentage, mean and standard deviation. Wherever necessary the results were depicted in the form of percentages.

RESULTS:

In this study 100 samples of BAL from chronic respiratory diseases cases were microbiologically analyzed for bacterial profile and the results are given below. The table1 shows the Age and Sex Distribution in Chronic Respiratory Diseases.

Table 1- Age and Sex Distribution in Chronic Respiratory Diseases

Age	Male	%	Female	%
< 20	1	2%	0	0
21-40	2	5%	13	26%
41-60	27	68%	24	47%
61 and above	10	25%	14	27%
Total	49		51	
Mean	10		12.75	
SD	12.02775		9.844626	

The age and sex distribution of the study population is shown in the above table. In this study female population had a slight increase in incidence 51% than male population which was 49 %. The commonest age group affected in the study was between 41 and 60. In this group male population was affected 68 % more than the female population which was only 47 % (Table.1). The fig.1 shows the Gender Distribution in Chronic Respiratory Diseases.

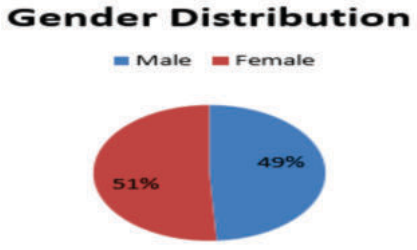

Fig. 1: Gender Distribution in Chronic Respiratory Diseases.

Table 2- Percentage of Gram Positive and Gram Negative Infection among Clinical Conditions

Diagnosis	Cases	G (+)	G (+) %	G (-)	G (-) %
COPD	27	6	38%	6	43%
Asthma	21	3	19%	1	7%
Chronic Bronchitis	30	4	25%	4	29%
Chronic Pleural Disease	6	1	6%	-	-
Emphysema	9	1	6%	3	21%
Pulmonary Fibrosis	6	1	6%	-	-
Cystic Fibrosis	1	-	-	-	-
Total	100	16		14	

G (+)- Gram positive; G (-)- Gram negative

Among the Chronic respiratory diseases the common occurrence was Chronic Bronchitis 30% followed by COPD 27% and Asthma amounting for 21%. Lower respiratory tract infection was observed more among COPD in which Gram negative organism was 43 % and Gram positive organism was 38%. This was followed by Chronic bronchitis in which Gram negative organism was 29 % and Gram positive organism was 25 %. Asthma cases had infection associated more with Gram positive organisms 19% than Gram negative organism 7% (Table.2). In our study chronic bronchitis accounts to 30%, followed by COPD 27% and Asthma 21% and chronic respiratory diseases.

Table 3- Profile of the Pathogens

Type of Organisms	No of Cases	(%)
Staphylococcus aureus	5	20 %
Acinetobactor baumanii	5	20 %
Enterococcus faecalis	2	8 %
Streptococcus pneumoniae	3	12 %
Pseudomonas aeuroginosa	1	4 %
Escherichia coli	2	8 %
Klebsiella pneumoniae	7	28 %
Total	25	

In this study a total of 25 organisms were isolated. Among bacteria gram negative organisms were isolated more than gram positive organisms. *Klebsiella pneumoniae* was the commonest bacteria isolated accounting to 28%. It was also noted that *Klebsiella pneumoniae* was also the commonest gram negative bacteria isolated. *Staphylococcus aureus* was the commonest positive bacteria isolated (20%).(Table.3).

Table 4- Distribution of Gram Positive and Gram Negative Bacterial Pathogens.

Types of Bacteria	Gram (+)	Gram (-)	Total No. Cases	%
Klebsiella pneumoniae	-	7 (47%)	7	28%
Acinetobacter baumanii	-	5(33%)	5	20%
Enterococcus faecalis	2(20%)	-	2	8%
Escherichia coli	-	2 (13%)	2	8%
Streptococcus pneumoniae	3(30%)	-	3	12%
Staphylococcus aureus	5(50%)	-	5	20%
Pseudomonas aeuroginosa	-	1(7%)	1	4%
Total	10	15	25	

The table 4 shows Gram negative infections are common in chronic respiratory disease which is 68% of bacterial infections. *Klebsiella pneumoniae* is the commonest among Gram negative bacteria followed by *Acinetobacter baumanii*. Gram negative bacterias were isolated more than Gram positive bacterias. The fig.2 shows Prevalence of Gram Positive and Gram Negative Organisms.

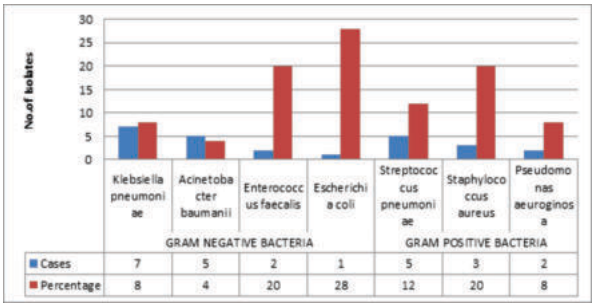

Fig.2: Prevalence of Gram Positive and Gram Negative Organisms

Table 5- Antibiotics Resistance Pattern Gram Negative & Positive Pathogens

Antibiotics	Klebsiella pneumoniae (7)	Acinetobater baumanii (5)	Escherichia coli (2)	Pseudomonas aeruginosa (1)	Staphylococcus aureus (5)	Streptococcus pneumonia (3)	Enterococcus faecalis (2)
Amikacin	0	1(20%)	1(50 %)	0	0	0	0
Amoxicillin/ Clavulanic	3(43 %)	3(60%)	2(100 %)	0	3(60%)	1(33%)	2(100 %)
Clindamycin	0	0	0	0	0	1(33%)	0
Cefoperazoine	1(14%)	2(40%		0	0	1(33%)	0
Cefepime	1(14%)	2(40%)		0	0	1(33%)	0
Imipenem	0	2(40%)	0	0	0	0	0
Linezolid	0	0	0	0	0	0	0
Vancomycin	0	0	0	0	0	0	0
Piperacillin /Tazobactam	0	1(20%)	0	0	0	0	0

Antimicrobial resistance is noted more among Gram negative bacterias than Gram positive bacterias in lower respiratory tract infections. There is a high amount of resistance to Amoxicillin/Clavulanic acid for both Gram positive and Gram Negative bacterias. Among all bacterias Pseudomonas aeruginosa shows least resistance and Acinetobacter baumanii shows highest resistance pattern (Table. 5).

Table 6- Age Prevalence among Different Pathogens

Antibiotics	Klebsiella pneumoniae	Acinetobater baumanii	Escherichia coli	Pseudomonas aeruginosa	Staphylococcus aureus	Streptococcus pneumonia	Enterococcus faecalis
1-30	0	0	0	0	0	0	0
31-60	5	4	1	0	4	0	1
61-90	2	1	1	1	1	3	1
Total	7	5	2	1	5	3	2

From this table 6 we observe that pulmonary infection are more common among 31-60 age group and less common among below 30 years of age. Bacterial infections are more common in the age group of 31-60 years with predominant Gram negative infections. In the age group above 60 years bacterial infections occurs equally with both Gram positive and Gram negative bacterias.

Polymerase Chain Reaction:

Bacterial colonies are formed and bacterial cells were lysed and genomic DNA was isolated. HELINI Ready to use *Klebsiella pneumoniae* Primer mix - 5□l/reaction PCR Product: 200bp The hemolysin gene was amplified from HELINI using forward (TTCATCTACGTGCTGGAGGG) and reverse (AGCCTGGATTGAGCGGATAA) primers. All the samples have been noted at 200 base pairs expected of *Klebsiella pneumonia* (Fig.3).

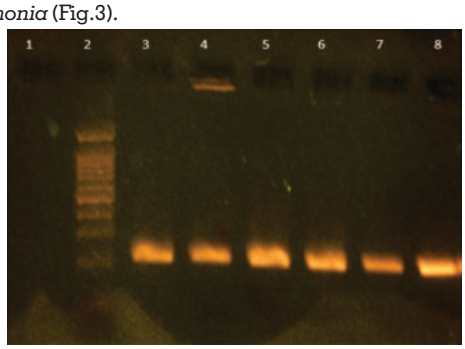

Fig.3: Gel Electrophorosis

Lane 1: Negative control. Lane 2- 100bp ladder. Lane 3-8- 200bp expected of *Klebsiella pneumoniae*.

DISCUSSION:

Bacterial infections are generally considered to be the most common cause of lower respiratory tract infection in chronic lung disease patients. Proper identification of respiratory pathogen from BAL is central for the treatment of pulmonary infections. Use of invasive techniques like bronchoscopic specimens like BAL fluid has led to better diagnosis of the lower respiratory tract infection. In non-ventilated patient, semi quantitative culture of BAL fluid has shown to have significiant predicative value for lower respiratory tract

infection. *Klebsillea pneumoniae* is one of the important Gram-negative bacteria in nosocomial infections. Antibiotic resistant isolates make therapeutic challenge in treatment of the patient. *Acinetobacter baumanni* is another Gram negative bacteria in hospital acquired infections and particularly pose problems due to large numbers of Multi drug resistant strains. This is a matter of great concern. Chronic respiratory diseases are public health problems due to their frequency of occurrence and financial impact on both developed and developing countries like ours. It is also a major contributor of motility and morbidity worldwide. Our study was conducted with the aim of determining the bacterial causative agents associated with chronic respiratory disease and also to estimate the antimicrobial sensitivity for the bacterial isolates. Chronic respiratory disease cases were more among the age group of above 40 years in our study which correlates with studies like *Mullerova et al* (45%) [7] and Merino- Sanchez et al (60%) [8] In lower respiratory tract infections BAL is a very useful diagnostic tool. This study showed a positive bacterial culture in 35 % of the cases of lung infection. This percentage is lower than when compared with studies like *Velez et al* (51.6%) [9], *Kottmann et al* (55.6%) [10] as they were done on immunocompromised patients. This study is in consistent with *Vivek KU* and *Nutan Kumar* study (38 %) [11] as both of the studies were carried out on general population. In our study, aerobic gram negative bacterias were 42 % and aerobic gram positive bacterias were 28 %. The percentages of gram negative bacterias are lower when compared with studies like Groenewegen and Wouters (71%) [12] with suspected pneumonia in COPD and *Vivek KU* and *Nutan Kumar* (65.7%) [11]. On the other hand our study is in consistence with gram positive bacterias 27% and 34.5% respectively. We observed klebisellia as the commonest pathogen isolated in this study which was 28%. This is in correlation with studies undertaken by *Lin SH et al* (19.6%) [13] and *Vivek KU* and *Nutan Kumar (*26%) [11]. Klebsiella being part of normal flora of mouth and commonly associated with pneumonia in elderly and hospitalized patients may been the reason of more isolation of the organism in our study. In this study Gram negative bacterias shows more antibiotic resistance when compared with Gram positive bacterias. Among Gram negative bacterisa *Acenitobacter baumanii* exhibits highest resistance followed by *Klebsiella pneumoniae* and *E. coli*. Least resistance was show by *Pseudonomas aeruginosa*. In Gram positive bacteria *Staphylococcus aureus* are resistant to Amoxicillin/clavulanic acid and exhibit sensitivity to other antibiotic. *Streptococcus pneumonia* shows 33% resistance to Amoxicillin/Clavulanic acid, clindamycin and cephalosporins. *Enterococcus exhibits* high resistance to Amoxicillin/clavulanic acid. All bacterial isolates were sensitive to Linezolid and vancomycin. Low number of bacterial isolates may be due to prior use of antibiotics. Conventional PCR test performed on samples of commonest isolate was tested for hemolysin gene for *Klebsiella pneumoniae* and were noted on 200 base pairs for *Klebsiella pneumoniae* in the post PCR product. PCR test was concordant with the phenotypic test.

CONCLUSION

Results from BAL fluid analysis made it evident that the colonizing microbial floras of the respiratory tract are responsible for infections in Chronic Respiratory diseases and are capable of frequently causing exacerbations. Gram negative bacterial infections are on the rise with high prevalence of drug resistance. Inappropriate choice of antibiotic and inadequate treatment poses a risk of Multidrug resistant pathogens which are difficult to treat and needs higher antibiotics which are expensive and not affordable to many. Institutional antibiograms are an important tool for detecting and monitoring antimicrobial resistance patterns among microbial pathogens. This can help in guiding the policy making decisions for the ethical use of antibiotics in treatment setting.

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