Study of bacterial microbiota in patients with acute exacerbation of bronchial asthma and chronic bronchitis

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Abstract

Introduction: Microbiota of respiratory tract may be responsible for acute exacerbation of bronchial asthma and chronic bronchitis. So the present study was carried out to find out specific bacterial etiological agents' involvement in acute exacerbation of these diseases.

Materials and Methods: Sputum samples from patients of bronchial asthma, chronic bronchitis during acute exacerbation and endotracheal aspirates from control cases were processed to isolate the microbiota of respiratory tract using quantitative methods after liquefying and diluting the samples. Significant and insignificant bacterial counts from the samples were determined. Grading of pus cells and bacterial morphology was seen by Gram's staining.

Results: Pus cell grading of 1+ to 3+ were seen in the both cases of bronchial asthma and chronic bronchitis whereas pus cells were not found in control cases. Percentage of microorganisms by Gram's staining in asthmatics, chronic bronchitis, and controls cases were 93.33%, 86.67%, 76.67% respectively. Among the pathogenic bacteria, percentage of significant count of *Staphylococcus aureus* and *Klebsiella pneumonia* were found to be high in chronic bronchitis cases when compared these pathogens in asthmatics. In bronchial asthma cases, the percentage of significant count of *Streptococcus pyogenes*, *Proteus mirablis* and *Pseudomonas aeruginosa* were high as compared to chronic bronchitis cases. In chronic bronchitis patients and asthmatics, significant count of non-pathogens were 16.67% and 13.33% respectively.

Conclusion: The quantitative cultures showing significant numbers as 10^4/ml and 10^7 /ml of pathogenic and nonpathogenic bacteria respectively are found to be associated with acute exacerbation of bronchial asthma and chronic bronchitis.

Keywords: Bronchial asthma, Chronic bronchitis, Microbiota, Quantitative culture.

Introduction

The human microbiota could be considered as the collection of bacteria, viruses, fungi, and archaea inhabiting in and on humans[1] The normal microbiota of several body parts have been described, including the gut[2] oral cavity[3] conjunctiva,[4] respiratory tract,[5] urogenital tract[6] skin[7] and blood.[8]

Oral microbiota are involved in upper and lower respiratory tract infections, which can also develop into atopic airway diseases such as allergic rhinitis and bronchial asthma[9] The oropharynx is constantly exposed to both inhaled and ingested microbes, including those contained in saliva.[10] It is known that the diversity of the oral microbiota can be affected by antibiotics, probiotics, diet, and gut microbiota.[11]

Chronic respiratory diseases, namely asthma, chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF), are among the leading causes of morbidity and mortality worldwide.[12] Oral microbiota is involved in conditions like bronchial asthma and COPD[13] since normal healthy lung is not a sterile organ as previously assumed.[14] Till date, the relationship between airway microbiota and chronic respiratory diseases has been investigated using sputum and bronchoalveolar lavage (BAL) samples[15-17] The clinical course of COPD is complicated by frequent episodes of acute exacerbation, which primarily involves microbial and viral infections.[18-20]

The common colonizers in COPD are nontypeable H. *influnzae* (60%), *M. catarrhalis* (48%) and 28% *S. pneumoniae.*[21] Moreover, bacterial colonization of the respiratory tract in COPD patients is dynamic, as the bacterial flora frequently changes in strains, species and number. The etiologic diagnosis of lower respiratory tract infection is complicated by the presence of harmless organisms, as well as that of potentially pathogenic organisms in the oropharynx. Colonization of the respiratory tract may be easily determined but differentiation of colonization from infection is difficult.[22] Sputum culture and gram staining are procedures to determine the number of potential pathogenic aerobic bacteria in respiratory secretions.[23,24]

Dixon and Miller (1965) investigated the value of diluting sputum before doing culture. They concluded that a routine dilution of 10^4 excluded the great majority of contaminating organisms and allowed a more meaningful report to be made.[25] Monroe et al (1969) quantitated microorganisms in sputum of a group of patients with pneumonia, and found that probable pathogens occurred in numbers of 10^7 organisms per ml or greater.[26] This work was confirmed and extended in 1969 by Pirtle, et al.[27]

Bacterial etiology in acute exacerbation with bronchial asthma and chronic bronchitis have been

studied by many researchers,[28,29] Dalvi et al in 1983 and Gaitonde in 1978 studied bacterial flora of sputum in exacerbation of bronchitis using criteria of significance as 10^7 colonies per ml for commensals and 10^4 colonies per ml for pathogens.[30,31]

The irregular distribution of pathogens in sputum makes direct or qualitative culture methods unreliable for incriminating any organism as pathogen. Quantitative culture method involving sputum liquefaction help in isolation of pathogenic bacteria without killing them.[23, 32]

The role of bacteria causing acute exacerbation in developing bronchial asthma and chronic bronchitis is significant and can be supported by the relief patients get from such acute exacerbation after the treatment of the underlying acute bacterial infections.

The present research study was undertaken to find out role of pathogenic bacteria causing acute respiratory tract infections and later acute exacerbation of asthma and chronic bronchitis, by using quantitative methods. The study of normal oral flora and its alteration in trachea in developing the acute exacerbation of asthma and chronic bronchitis was also investigated in this research study.

Materials and Methods

The present study was done in Department of Microbiology, PMCH, Udaipur which is a continuation of previous study done in Department of Microbiology, Dr. S.N. Medical College, Jodhpur. Bacteriological study of sputum samples was conducted in 30 cases of bronchial asthma and 30 cases of chronic bronchitis during acute exacerbation and 30 control cases. The patients of bronchial asthma and chronic bronchitis were selected as per the standard guidelines. Control cases having no history of upper or lower respiratory tract infection and going for surgery were selected.

Investigations

1. Peak expiratory rate
2. Sputum examination- Morning sputum was collected in sterile petri-dish from cases after mouthwash with lukewarm water. Macroscopic examination, Leishman staining for pus cells and eosinophils, Gram staining of sputum for presence of bacteria, and quantitative culture after liquefaction and appropriate dilutions were carried out.

Leishman Staining for Pus cells and Eosinophils: Sputum smear was made from purulent part and stained with Leishman staining as described by Dacie & Lewis.[33] Microscopic purulence was graded depending upon pus cells per oil immersion field as described by McHardy et. al.[34]

Gram Staining of Sputum: Sputum was assessed for suitability of culture by determining number of squamous epithelial cells, pus cells and/or bacteria.[35] The smear was made from most purulent part of sputum.

Liquefaction of Sputum Sample: To one volume of sputum sample, five volumes of physiological saline was added. This mixture was well shaken to free it from most of the adherent saliva and saline solution was removed with pasture pipette. Liquefaction of sputum was done by adding 2 ml of 2% N acetyl –L-cysteine to the equal amount of sputum-saline mixture and shaken at room temperature for five minutes as described by Wilson & Martin 1972 with partial modification.[23]

Quantitative Culture of Sputum: Quantitative culture of sputum sample was done by standard loop method.[36] The liquefied sputum was diluted to various dilutions from 1:1, 1;10, 1:100 to 1:1000 in peptone water for obtaining isolated bacterial colonies. Before inoculation on suitable culture media for quantitative culture, liquefied sputum was well shaken. All diluted samples were inoculated on blood agar, chocolate agar and thioglycollate liquid medium by using previously standardized loop having aliquot holding capacity of 0.005 ml. Plates were incubated in CO_2 enriched atmosphere. After 24 to 48 hours incubation, identification of bacteria was made as per colony characters and biochemical reactions.[36] Significant bacterial count for pathogenic bacteria and nonpathogenic bacteria or commensalshas been considered as 10^4/ml and10^7/ml respectively in the present study.[25,31] Bacteria per ml of sputum was calculated by following formula:

$$Q = \frac{C \times DF}{AQ}$$

Where,

Q= Number of bacteria/ml of sputum or Endotracheal aspirate.

C=Number of colonies per plate

DF= Dilution Factor

AQ= Loopful aliquot

Endotracheal Aspirate (EA) Culture: The, samples were collected prior to tracheal intubation through laryngoscope, one for Leishman and gram's staining and another for quantitative culture. For quantitative culture EA was serially diluted in sterile normal saline as 1/10, 1/100, 1/1000 and 0.01 ml of 1/1000 dilution was inoculated on 5% sheep blood agar. After incubation at 37ºC in CO_2 incubator for 24 h, colony count was done. The number of bacteria /ml of EA was calculated. (Fig. 1).

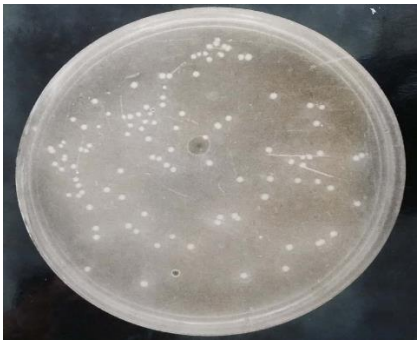

Fig. 1: Quantitative culture of sputum showing colonies of *Staphylococcus aureus* on NA

Results

Patients from 21 to 70 years were studied with male predominance (73.33%). Most of asthmatic patients (83.33%) belonged to age group 41 to 60 years. The percentage of chronic bronchitis cases (93.33%) was highest in age group 21 to 60 years with male predominance (66.67%). The 83.33% control cases were in age group 31 to 60 years with male controls 60% and female controls 40%. The total leukocyte count varies from 8,000 to 18,000/cumm in both asthmatic patients and chronic bronchitis patients. The range of TLC for all control cases was from 4500 to 11000/cumm. In 53.33% cases TLC was raised in asthmatics whereas higher percentage (60%) of TLC was noted for chronic bronchitis cases. The raised absolute eosinophil count 30%, 3.33% (above 440/cumm) were noted in asthmatics and chronic bronchitis cases respectively. None control cases had raised blood eosinophilia. In all asthmatics, peak expiratory flow rate was diminished (50 to 250 liters per minutes) as compared to normal (440 to 550 liters/minute).

Sputum eosinophilia 11% and 14% was noticed in two cases of asthma during acute exacerbation (6.67%) cases. These cases had blood eosinophilia too. In none control cases had raised sputum eosinophilia. Chronic bronchitis showed only one case of blood eosinophilia (3.33%) that was without sputum eosinophilia.

Table 1 Shows pus cell grading and bacteria in grams staining in acute exacerbation of bronchia l asthma and chronic bronchitis and control cases. Pus cell grading was 1 + and 3+ in 30% cases, and 2+grading in 40% cases of bronchial asthma. Chronic bronchitis cases showed 50% cases in 1+, 23.33% cases in 2+ and 26.67% cases in 3+ pus cell grading. In control cases no pus cells were seen. The different morphological form of bacteria seen in 28 cases (90%) in asthmatics, in 26 cases (86.67%) in chronic bronchitis cases and 23 control cases (76.67%).

Table 2 Shows different types of bacteria isolated along with their viable counts from sputum in cases of acute exacerbation of bronchial asthma and chronic bronchitis and controls cases. The number of bacteria isolated is high than total number of cases because of one or more than one type of bacteria was isolated from single sample in few cases. In the pathogenic group, the percentage of significant viable bacterial counts of *Staphylococcus aureus* and *Klebsiella pneumoniae* was high in chronic bronchitis cases (30% and 16.67%) as comparison to bronchial asthma cases (16.67, 10%) during acute exacerbations. Isolation of *Streptococcus pyogenes* (10%), *Proteus mirablis* (10%), *Pseudomonas aeruginosa* (6.67%) was at higher number in bronchial asthma cases when compared to chronic bronchitis cases i.e. 3.33%, 0% and 3.33%. Significant viable count of *E. coli* recovered only from chronic bronchitis 3 cases (10%) and only one case (3.33%) from asthmatics. *Streptococcus pneumoniae* (6.67%) revealed significant viable count in chronic bronchitis cases and in insignificant number (3.33%) from asthmatics. Among non pathogenic bacteria, *Streptococcus viridians* (10%), *M. catarrhalis* (3.33%) and *Micrococcus* (3.33%) were also isolated in significant number in chronic bronchitis cases. These bacteria were also isolated from asthmatics in significant number. From one case Non enterococcus D was also isolated in significant number from asthma cases. Control cases in majority of cases showed the presence of non pathogenic bacteria like *Streptococcus viridians* (33.33%), *M. catarrhalis* (13.33%), *Staphylococcus albus* (13.33%) and *Micrococcus* (3.33%) in insignificant number. Among pathogenic bacteria *Streptococcus pnuemoniae* was recovered in insignificant number. None of control cases showed significant viable bacterial count.

Table 1: Pus cell grading and bacteria in grams staining in acute exacerbation of bronchial asthma and chronic bronchitis and control cases

Bronchial Asthma Gram's Staining(30 cases)							**Ch. Bronchitis Gram's Staining(30 cases)**						**Control Cases Gram's Staining (30)**	
Bacteria Seen in Gram staining	**Pus cell**						**Pus cell**						**Pus cell**	
	1+		2+		3+		1+		2+		3+		**Pus cell not seen**	
	No	%	No	%	No	%	No	%	No	%	No	%	No	%
GPC groups	1	3.33	2	6.67	3	10.00	2	6.67	3	10.00	3	10.00	3	10.00
GPC pairs	1	3.33	2	6.67	-	-	1	3.33	1	3.33	-	-	2	6.67
GPC Chains	-		2	6.67	1	3.33	1	3.33	1	3.33	1	3.33	5	16.67
GNC	1	3.33	1	3.33	1	3.33	2	6.67	-	-			6	20.00
GNB	1	3.33	2	6.67	1	3.33	3	10.00	1	3.33	3	10.00	-	-
Others	-	-	1	3.33	-		1	3.33	-	-	-	-	1	3.33
one or >one type	3	10.0	2	6.66	3	10.00	2	6.67	-	-	1	3.33	6	20.00
Total	7	23.3	12	40.0	9	30.0	12	40.00	6	20.00	8	26.67	23	76.67
Bacteria not Seen in Gram staining	2	6.67	-	-	-	-	3	10.00	1	3.33	-	-	7	23.33
Grand Total cases	9	30.0	12	40.0	9	30.0	15	50.00	7	23.33	8	26.67	30	100.00

Table 2: Bacteria and viable counts from sputum in cases of acute exacerbation of bronchial asthma and chronic bronchitis and controls cases

Bacteria Pathogenic Bacteria	Bronchial asthma cases(30)				Chronic bronchitis cases(30)				Control cases (30)			
	Significant viable count		Insignificant viable count		Significant viable count		Insignificant viable count		Significant viable count		Insignificant viable count	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Staph. aureus	5	16.67	-	-	9	30.00	-	-	-	-	-	-
Klebsiellapneumoniae	3	10.00	-	-	5	16.67	-	-	-	-	-	-
Strept. pyogenes	3	10.00	-	-	1	3.33	-	-	-	-	-	-
Proteus mirablis	3	10.00	-	-	-	-	-	-	-	-	-	-
Esch. coli	1	3.33	-	-	3	10.00	-	-	-	-	-	-
Pseudomonas aeruginosa	2	6.67	-	-	1	3.33	-	-	-	-	-	-
Strept. pneumoniae	1	3.33	1	3.33	2	6.67	1	3.33	-	-	1	3.33
Non-enterococcus D	1	3.33	-	-	-	-	-	-	-	-	-	-
Total	19	63.33	1	3.33	21	70.00	1	3.33	-	-	1	3.33
Non Pathogenic Bacteria												
Strept. viridans	2	06.67	4	13.33	3	10.00	5	16.67	-	-	10	33.33
M. catarrhalis	1	03.33	5	16.67	1	3.33	1	03.33	-	-	04	13.33
Staph. albus	-	-	2	6.67	-	-	1	03.33	-	-	6	20.00
Micrococcus	1	03.33	1	03.33	1	3.33	4	13.33	-	-	2	06.67
Total	4	13.33	12	40.00	5	16.67	11	36.66			22	73.33
Grand Total	23	76.67	13	43.33	26	86.67	12	40.00	0.00	0.00	23	76.67

Discussion

Most of asthmatic patients (83.33%) belonged to age group 41 to 60 years. The percentage of chronic bronchitis cases (80.00%) was highest in age group 31 to 60 years. The incidences of both diseases were in middle and old age may be due to lowered immunity, underlined disease and age factor making these patients susceptible to bacterial infections. Although all cases of bronchial asthma had purulent sputum, leukocytosis was noticed in 64% only indicating that it is not an absolute indicator of infection in cases of bronchial asthma. Chodosh (1987) also noticed mild leukocytosis in these patients.[29] Bronchial asthma is usually associated with eosinophilia.[37] However in the present study, absolute eosinophil counts of blood exceeded only in nine cases above normal (440/cmm) suggesting that in infective bronchial asthma, eosinophilia is not always found. This may be due to stimulation of production of neutrophils by infective agents whose production exceeds that of eosinophils. The later may not be produced excessively due to substrate competition. Sputum eosinophilia was seen only in two patients (6.67%) suggesting that major pathogenesis in lung is infective rather than allergic aetiology. There was no correlation between blood and sputum eosinophilia. Similar were the observations of McHardy et al (1980).[34] Blic et al (1982)[38] had also observed increased pus cell with decreased eosinophils during acute exacerbation of bronchial asthma. They also found that after therapy, the pus cell decreased and eosinophils increased. The assumption that major pathology in the present study was infective is also supported by the observation of blood cells of chronic bronchitis cases with acute exacerbation where similar finding was noticed.

Examination of sputum smears stained with grams stain revealed microorganisms in 93.33% of bronchial asthma cases, 86.67% in chronic bronchitis cases with acute exacerbation. However in control cases, microorganisms were found only in 76.67% of cases. Morphology of bacteria seen during gram staining also confirmed by culture in most of the cases. These observation suggests that examination of sputum stained with gram staining can reveal to a great extent whether one is dealing with infective or non- infective cases. The number of pus cell in smear can give a clue to severity of infection. Majority of cases of bronchial asthma the pus cells grading, + and ++ were seen whereas in chronic bronchitis cases, the pus cell grading was +++ in majority of cases. These observation suggests that infection in bronchial asthma was of mild to moderate degree and in chronic bronchitis cases it was of severe grade. The association of pus cell and bacteria in a sputum smear provides a definite index of pulmonary infection. Therefore, sputum examination must always be performed along with the culture studies. At a time the report of sputum smear can be a guide line to start the treatment as the report of culture can be delayed by 48 hours.

The observation on culture study of sputum isolated two groups of organisms one belonging to commensals of upper respiratory tract, which is also found in tracheal aspirates of healthy control cases and other to pathogenic group... In the present study predominant pathogenic organisms recovered in significant number (10^4/ml) in 63.33%of cases from asthmatics and were *Staph. aureus*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Strept. pyogenes*, *Psuedomonas aeruginosa*, *Strept. pneumoniae*, Non enterococcus group D and from commensals in significant number (13.33%) were *Strept. viridans*, *M. catarrhalis* and *Micrococcus* The majority of pathogenic bacteria isolated in significant number (70%) from chronic bronchitis cases were *Staph aureus*,

Klebsiella pneumoniae, E. coli and *Strept. pnuemoniae*. Amongst non-pathogenic bacteria (16.67*%) Strept viridans, M. catarrhalis* and *Micrococcus* were isolated in significant number (10^7/ml). These bacteria were also isolated from asthmatics in significant number. Various studies by different authors e.g. Lambert & Stern 1972,[28] Hudgel et al. 1979,[40] Chodosh (1987),[29] HsuAC et al 2015 & 2016[19,20] showed varied results in the isolation of various pathogenic and commensal bacteria. Valencia AM et al.(2003) from a multicentric study from Spain concluded that the quantitative cultures of EA can be considered acceptable for the diagnosis of VAP[39] The present study had revealed that majority of infective agents of two conditions vary in proportion of isolation not only with each other but also in respect to commensals. These differences in bacterial culture may be due to the fact quantitative culture of sputum was not done in few of the studies and secondly the differences may be related due to use of antibiotics in the community during and before the study. Indiscriminate use of antibiotics has resulted in colonization of drug resistance organisms which explain higher isolation of these organisms.

The P-value, 3.673e-05, calculated by Chi-square, is less than 0.5; this shows that the number of pathogenic and non-pathogenic bacteria is not independent of their frequencies in type of pulmonary infections. In other words, there is association of number of pathogenic as well as non-pathogenic bacteria with a particular type of pulmonary infection. Although the insignificant viable count of commensals were higher but significant viable count of these commensals cannot be overlooked as justified by studies of Ramsey et al. 2007[13] and Bourdine et al 2009.[9] These authors also emphasized on role of oral microbiota. According to these authors oral microbiota are known to be involved in upper and lower respiratory infections, which can also develop into atopic airway diseases such as allergic rhinitis and bronchial asthma. Therefore these can trigger and strike as a critical component in conditions like bronchial asthma and COPD. Therefore not only non- pathogenic bacteria isolated from sputum showing significant viable count may cause lower respiratory infections during acute exacerbations but the role of bacteria with insignificant viable count causing lower respiratory infections in immunocompromised patients cannot be ruled out.

Conclusion

The cases of bronchial asthma and chronic bronchitis with acute exacerbations were seen in middle age group. During acute exacerbations in bronchial asthma blood eosinophilia is not always found. The number of pus cell in sputum smear can give a clue to severity of infection. Morphology of bacteria seen during gram staining also confirmed by culture in most of the cases. The quantitative cultures showing significant numbers as 10^4ml and 10^7/ml of pathogenic and nonpathogenic bacteria respectively are found to be associated with acute exacerbation of bronchial asthma and chronic bronchitis in the present research study.

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