

Evaluation of Atypical Bacteria Involved in Recurrent Deterioration of Symptoms in Chronic Obstructive Pulmonary Disease

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ABSTRACT

OBJECTIVE: To evaluate the bacterial colonies involved in the recurrent deterioration of symptoms in chronic obstructive pulmonary disease (COPD) patients.

METHODOLOGY: This is an observational cross-sectional study. This study was carried out at the outpatient department (OPD) and from inpatients who were admitted as an inpatient in Medical & Pulmonology wards due to severity of illness in Lyari General Hospital from March to September in 2018. Ethical approval was taken from SMBB Medical College Karachi. 120 diagnosed COPD patients with regular follow-up in OPD with written consent, and aged between 40 to 65 years from both sexes were included by non-probability consecutive sampling technique, and Subjects with age less than 40 years, Known case of allergy, asthma, on prophylactic antibiotic therapy were excluded. All information was collected on the preformed proforma. Sputum samples were collected to Dow laboratory for culture and DNA extraction. All samples were analyzed microscopically and via Polymerase chain reaction (PCR). Statistical analysis was performed by SPSS 22.0.

RESULTS: 60 stable state samples and 60 aggravated condition samples were collected from subjects on treatment for COPD. Stable state samples were negative for *Chlamydophila pneumoniae*, *Mycoplasma pneumoniae*, *Streptococcus*, and *Haemophilus* DNA, however, samples showed the presence of *Moraxella* (n=15), *Nocardia* (n=3), *Lactobacilli* (n=5), and *Legionella* (n=1) DNA. In aggravated state samples *Haemophilus influenza* (n =11), *staphylococcus aureus* (n=8), *staphylococcus pneumonia* (n=9), *E.coli* (n=8), *lactobacillus* (n=7), *Bifidobacteria* (n=7), *Nocardia* (n=10).

CONCLUSION: There is a highly unique relationship between atypical bacteria and recurrence of the severity of symptomatology in COPD.

KEYWORDS: Atypical Bacteria, COPD, *Moraxella*, *Nocardia*, *Lactobacilli*, *Bifidobacteria*.

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INTRODUCTION

Chronic obstructive pulmonary disease (COPD) or chronic obstructive airway disease (COAD) is a type of obstructive airway lung disease and it is associated with progressive inflammation in the lung and is characterized by breathing problems with poor air entry¹. It is considered as a preventable and treatable disease with persistent respiratory clinical features of obstruction in airflow or breathing.

It is estimated that 16 million people are suffering from COAD in the United States of America; the incidence of chronic obstructive airway disease is increasing in patients with regular smoking habits or having a history of raw tobacco consumption with age above 40 years, and in the male gender. COPD is defined as being a "stable" and "unstable" condition based on the progress of the disease².

In developing countries, common sources are domestic use of biomass fuel³. These irritants in long-term exposure cause inflammation in the lungs with

bronchoconstriction of small lung airways and destruction of the lung parenchyma. Chronic obstructive airway disease (COAD) is considered as a leading factor that affects the health status, socioeconomic status, and poor outcome in quality of life (QOL), that ultimately responsible for the higher incidence of morbidity and mortality worldwide⁴.

Acute deterioration or acute exacerbations of COPD (AECOPD) is associated with multiple hospital admissions, worsening of health care cost, or increased economic burden on sufferers⁵. The AECOPD means worsening of symptoms with deterioration pulmonary functions, which mostly occurs due to infections or smoke and air pollution. It ultimately leads to acute respiratory failure as a life-threatening complication⁶.

The process of progression of respiratory tract inflammation (RTI) by microorganism and clinical features in chronic obstructive airway disease (COAD) remains unclear while using bronchoalveolar lavage fluid and sputum in the diagnosis of potentially

pathogenic microorganisms (PPMs) in AECOPD. The *Haemophilus influenzae* and *Moraxella catarrhalis* are commonly identified microorganisms and their association with bacterial infection, which leads to worsening of health, QOL, and AECOPD with the inflammatory process⁷. A sensitive as well as specific molecular technique, like the Polymerase chain reaction technique can be used for evaluating the atypical bacteria. Polymerase chain reaction (PCR) can detect tiny fragments and small fragments of nucleic acids of all pathogens; it is not dependent upon the capability of the target microbe; as it is possible that it can be affected by earlier antibacterial treatment⁸.

The relationships between airway inflammation and PPMs, these PPMs can be evaluated by quantitative real-time polymerase chain reaction (qPCR or RT-PCR) in bronchial secretions (sputum) from AECOPD patients, although how this technique is unclear about culture and clinical measures of the disease⁷. Hence it can be hypothesized that the recurrence of the severity of symptoms is due to the presence of PPMs. These can be detected from the sputum samples of both stables as well as acutely ill (unstable) chronic obstructive airway disease (COAD) patients by using RT-PCR. The objective of this study was to evaluate the bacterial colonies involved in the recurrent deterioration of symptoms in COAD patients by RT-PCR.

METHODOLOGY

This was a cross-sectional study performed in the outpatient department (OPD) and from inpatients who were admitted inwards (Medical & Pulmonology) due to severity of illness at Sindh Government Lyari General Hospital from March to September in 2018. Ethical approval was taken from the department of postgraduate Medical education, training, Research & Shaheed Mohtarma Benazir Bhutto Medical College Lyari Karachi. One Hundred Twenty diagnosed COAD patients and regular follow up in outdoor patient department with informed and written consent aged between 40 to 65 years from both sexes were enrolled by non-probability consecutive sampling technique were enrolled, and Subjects with age less than 40 years, Known case of Allergy or atopy or asthma or on treatment were excluded. All the data were collected on proforma.

All subjects were grouped equally into Group-A and Group-B. Group-A with 60 randomly selected COAD patients with acute severity of symptoms, the sputum samples of this group are marked as aggravated state sputum. Group-B with 60 COAD patients were not having acute severity of clinical features and their sputum production samples are marked as stable state sputum. Naturally coughed out sputum was

collected in sterile bottles and procedure was done for culture and DNA extraction within 4-5 h. Sputum samples were collected and sent to Dow university laboratory for culture and DNA extraction. All samples were analyzed microscopically and via Polymerase chain reaction (PCR).

Sample Analysis & RT-PCR Technique; All the sputum samples were incubated at temperature i.e 37°C for 15 min and 0.1% dithiothreitol was added. All the sputum samples were divided into two equal portions with one part utilized for gram staining sputum samples and was observed microscopically. Each to be considered as representative bronchial sample must have $<10^5$ epithelial cells·mL⁻¹ which is equal to <1 epithelial cell per high-power field. 200 µL sputum sample was collected by using NucliSens® easyMAG™ (bioMérieux, USA)⁹. According to the manufacturer's instructions, 5 µL sputum samples were utilized as a PCR template. DNA was stored at -20°C till PCR was done. For the identification of *Haemophilus influenzae*, *Staphylococcus aureus*, and *Staphylococcus pneumoniae* an assay was performed using 2 and 4 outer membrane protein, variable nucleotide sequence. Identification of *E.coli*, *Lactobacillus*, *Bifidobacteria*, *Legionella*, and *Nocardia* two different assays were utilized directed on explicit zones of 5S ribosomal ribonucleic acid (rRNA) with macrophage infectivity potentiator (*MIP*) gene. The PCR-probe assay for *E.coli*, *Lactobacillus*, *Bifidobacteria*, *Nocardia*, and *Legionella*, were done on the primers described by Findlay *et al.*¹⁰ and Leg 5S detected in real-time using a TaqMan probe, Netherlands. Another PCR was performed to detect *Haemophilus influenzae*, *Staphylococcus aureus*, and *Staphylococcus pneumoniae*, the PCR was based on the sequences of the *MIP* gene, P1 adhesion gene assay. 66 base pairs amplicons were extracted using forward primers 5'-TGG TAA CTG CCC CAC AAG C-3' and 5'-GGT CAA TCT GGC GTG GAT CT-3'. The Fluorescent labeled 6-carboxyfluorescein Taqman probe 5'-TCCCCC GTT GAA AAA GTG AGT GGG T-3' was used for RT-PCR. All the samples were processed for DNA extraction using both Qiagen (Proteinase K and Buffer AL from the Qiagen DNA easy Blood and Tissue kit) and microLYSIS techniques. This was done to detect every possible available bacterial DNA present in the sample and avoid missing microbiota.

RESULTS

A total of 120 samples were collected from 120 patients provided. 60 spontaneously expectorated sputum samples from each group. **Table I.** Shows all participants were divided into 2 groups (A & B) based on sputum production. In group A (n= 60) male participants were 35 in which mean age was 55 years.

All are factory workers/Skilled Labors, ghutka, smokers, Occupational and therapeutic History (Non-Compliant / taking symptomatic treatment). In the same group, remaining female participants (n = 25), mean age was 50 years, housewives, having a history of ghutka, smoking, and Biomass Fuel stove with therapeutic History (Non-Compliant/ taking symptomatic treatment). In group A (n= 60) male participants were 31 in which mean age was 50 years. All are factory workers/Skilled Labors, ghutka, smokers, and therapeutic History (On regular treatment + follow-up). In the same group, remaining female participants (n = 29), mean age was 42 years, housewives, having a history of ghutka, smoking and Biomass Fuel stove with therapeutic History (On regular treatment + follow up).

DNA Detection: **Table II** shows the comparison between DNA detection capacity of both Qiagen and microLYSIS, in the table, it can be seen that the capacity of the microlysis technique towards detection of atypical bacteria is slightly better than Qiagen. However, the study was unable to identify any major differences in the detection of atypical bacteria between the both.

Atypical Bacterial Load in both groups: Graph I shows on its x-axis the gender-wise group distribution while the y-axis shows the log (CFU/ml & Genome/ml). The different color bars show the type of bacteria being identified in each group. In group A (aggravated state sputum) male gender showed a high presence of Nocardia along with, Homophiles, E.coli, Moraxella, bifidobacteria, and lactobacillus (as per highest to lowest order). While in group A (aggravated state sputum) female gender showed Nocardia along with, Moraxella, Homophiles, E.coli, lactobacillus, streptococcus, and bifidobacteria (as per highest to lowest order). In group B (Stable state sputum) male gender showed a high presence of streptococcus along with, Homophiles, Moraxella, E.coli, Nocardia, bifidobacteria, and lactobacillus (as per highest to lowest order). While in group B (Stable state sputum) female gender showed Homophiles in the highest

percentage along with E.coli, Moraxella, Nocardia, bifidobacteria, and lactobacillus. (As per highest to lowest order).

Table III showing a comparison of bacterial (Typical/ Atypical) detection capability of Culture and Polymerase chain reaction (PCR) techniques it can be seen that the detection capacity of simple culture techniques is less than that of PCR. 60 samples of group A (Aggravated State Sputum) the efficacy of culture techniques were 18/60 (30%) in comparison with Polymerase chain reaction (PCR) having detection capacity of 45/60 (75%). The results depict that PCR is far more superior to conventional culture techniques. In group B (Stable State Sputum) the detection capacity of the culture technique was 27/60 (45%) on the other hand Polymerase chain reaction (PCR) was able to detect 40/60 (66%). On comparing results of both groups it can be analyzed that the PCR method has better detection capacity as compared to conventional culture techniques.

TABLE II: COMPARISON B/W QIAGEN AND MICROLYSIS DNA BACTERIAL DETECTION IN CHRONIC OBSTRUCTIVE AIRWAY DISEASE (COAD)

Pure Cultures	QIAGEN	MicroLYSIS
Haemophilus	2.144E+8	2.490E+9
Moraxella	1.899E+8	2.011E+9
Nocardia	1.759E+8	2.595E+9
E. coli	1.251E+8	2.112E+9
Lactobacilli	2.679E+8	3.214E+8
Streptococcus	3.203E+8	2.114E+8
Bifidobacteria	2.858E+8	2.254E+7

E=Exponent, DNA was extracted in triplicates from each pure culture. 16 S q PCR assays were performed to enumerate the genome /ml readings from the extracted DNA C.F.U/ml counts were performed

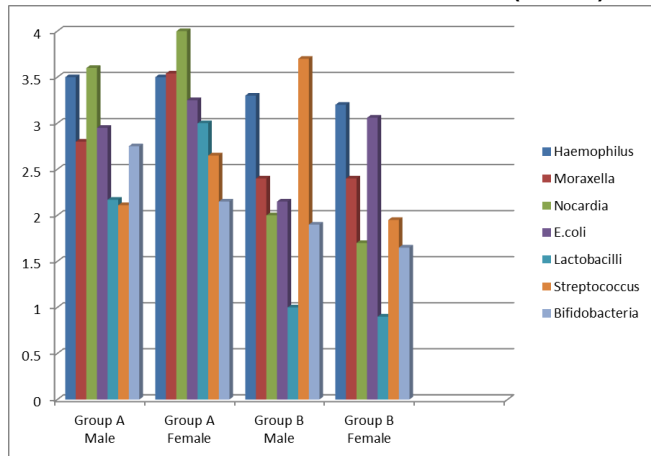
TABLE I: GENERAL CHARACTERISTICS OF CHRONIC OBSTRUCTIVE PULMONARY DISEASE (COPD)

GROUPS	Gender	Mean Age (Years)	Occupation	Past personal Habits / Occupational History	Therapeutic History
Group-A Aggravated State Sputum (n=60)	Male (n= 35)	55	Factory workers/ Skilled Labors	Smoker + Occupational	Non Compliant / Symptomatic treatment
	Female (n= 25)	50	Housewives	Gutka + smoking + Biomass Fuel stove	Non Compliant/ Symptomatic treatment
Group-B Stable State Sputum (n=60)	Male (n= 31)	50	Factory workers/ Skilled Labors	Gutka + smoking	On regular treatment + follow up
	Female (n= 29)	42	Housewives	Gutka + smoking + Biomass Fuel stove	On regular treatment + follow up

TABLE III: COMPARISON OF BACTERIAL (TYPICAL/ATYPICAL) DETECTION CAPABILITY OF CULTURE AND PCR TECHNIQUES IN CHRONIC OBSTRUCTIVE PULMONARY DISEASE (COPD)

GROUPS	CULTURE (n=120)	PCR (n=120)
Group-A Aggravated State Sputum (n=60)	18/60 (30%)	45/60 (75%)
Group-B Stable State Sputum (n=60)	27/60 (45%)	40/60 (66%)

GRAPH I: PERCENTILE OF ATYPICAL BACTERIA DETECTED IN BOTH GROUPS IN CHRONIC OBSTRUCTIVE PULMONARY DISEASE (COPD)



X Axis; Groups Division gender wise, Y Axis; log(CFU/ml & Genome/ml)

DISCUSSION

Frequent exacerbation of COPD/COAD increases the economic burden (50-75%) on the health care system of any country or society and globally as well, and it is reflected by an increase in the admissions or hospitalizations and frequent visits of OPD^{11,12}. The recurrent Respiratory infection is a major cause of exacerbation in COPD or COLD patients. It is an astonishing observation in this study that Nocardia became the predominant pathogen log/ml in both sexes of group suffering from acute exacerbation of symptoms; another important finding in the same group is Haemophilus, Moraxella and E.coli were also present in high percentage log/ml in the acute exacerbation group. Lactobacilli and Bifidobacteria were also identified in more than normal percentage log/ml in the acute exacerbation group. These findings indicate a probable positive association between recurrent acute exacerbations of symptoms of COPD in our population, which are consistent with the findings of Fraser DW et al¹³.

The COPD is considered as a risk factor for the development of Pulmonary Nocardiosis (PN)¹⁴. Various pathogens such as bacterial streptococcus species especially pneumoniae and Moraxella catarrhalis along with viruses have been proven to play an important role in the exacerbation of COPD symptoms by Bartlett JG, et al¹⁵. However the current study shows a high presence of atypical bacteria in the targeted population, while Streptococcus pneumoniae and Moraxella Catarrhalis were also present in high percentage. The findings may be variable from Bartlett JG, et al¹⁵ could be due to the variation in techniques that is in the current study we used 16S DNA sequencing while Bartlett JG, et al¹⁵ used simple culture techniques. Another reason could be the variation in personal habits of the study population. These pathogens are strongly correlated with inflammation and it leads to an increase in the pro-inflammatory markers. These mediators like enzymes (proteases), chemokines, oxidative stress-related molecules, cytokines secreted in the sputum (IL-1 β , IL-10, and tumor necrosis factor (TNF)- α) and decreased CCL13 and blood cells like eosinophils and macrophages play important role in phagocytosis¹⁶.

The Potentially pathogenic microorganisms (PPMs) in COAD patients had a decreased phagocytic activity, which leads to an increase in the persistence or worsening of the inflammation that affects or decreases the clearance of bacterial and viral pathogens¹⁷. In COPD or COAD basic fibroblast growth factor bFGF) or Fibroblast growth factor 2 (FGF2) are overexpressed particularly in patients with AECOPD via vasodilatation in the inflammatory process and having a history of smoking¹⁸. In the current study it is well established finding that in the acute exacerbation phase atypical bacteria for the respiratory tract such as Bifidobacteria, Morexella, Ecoli, and Lactobacillus were identified in the group with recurrence of acute exacerbation of symptoms in both sexes. This is persistent with findings of Raty R 1999¹⁹. The main mechanism which can be postulated after extensive literature search is the use of random medications and antibiotics along with a non-quantified dosage of steroid-based medications. It has been observed that one of the major issues in the non-uniformity of data is due to segregated methodology for the identification of pathogens. This lack of standardization has led to a variation of published data concerning COPD.

On comparing different DNA extraction techniques, the most commonly used techniques were Qiagen and microLYSIS. Both techniques were compared in the current study (Table I). It was observed that both techniques have almost the same efficacy in the identification of typical as well as atypical microbiota. These findings are in contrast with the findings of Donaldson GC 2002²⁰, however almost similar

efficacy of Qiagen and microLYSIS were observed by Blasi F et al²¹.

When conventional culture techniques were analyzed and compared with RT-PCR. It can be observed from the given data that the accuracy of RT-PCR is quite superior to conventional plate techniques these observations are quite following the findings of Murphy TF et al²², and no differentiation was observed in the capacity of both the techniques.

CONCLUSION

As it is intricate to convincingly conclude that the presence of atypical bacteria is the main reason for the recurrence of symptoms but an indirect perspective can be drawn from the current findings. It can also be concluded that the technique for prescribing random antibiotics to patients of COPD without proper culture and sensitivity can be one of the reasons for atypical presence.

RECOMMENDATIONS

To understand the diversity of atypical microbiota. It is important to utilize sensitive advanced techniques for routine investigations in the detection of bacterial communities in the COPD population.

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AUTHOR CONTRIBUTIONS

Rashid MN: Concept of study

Laghari Z: Critical review

Shahid R: Data collection

Abro SA: Writing manuscript

Saleem Q: Data analysis

Noman B: Data collection

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