ORIGINAL ARTICLE

SPUTUM CULTURE AND POLYMERASE CHAIN REACTION TESTS FOR IDENTIFICATION OF BACTERIAL PATHOGENS IN EXACERBATION OF CHRONIC OBSTRUCTIVE PULMONARY DISEASE

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Background: Chronic Obstructive Pulmonary Disease (COPD) is a growing pulmonary disorder comprised of chronic bronchitis and emphysema. This condition is characterized by breathlessness, decrease immunity, and recurrent respiratory tract infections (RTI). The objective of this study was identification of predominant bacterial pathogens in respiratory tract, responsible for acute exacerbation of chronic obstructive pulmonary disease (COPD) patients using highly sensitive real-time Polymerase Chain Reaction (PCR) and conventional methods. **Methods:** The retrospective study was conducted in tertiary care hospitals of Karachi, 120 diagnosed regular follow-ups of chronic obstructive pulmonary disease (COPD) patients with informed and written consent aged between 40–55 years from both sexes were included in the study. All samples were analyzed through sputum culturing and PCR assays. **Results:** Sixty stable state samples and 60 aggravated condition samples were collected from subjects on treatment for the chronic obstructive pulmonary disease (COPD). Stable state samples were negative for Chlamydophila pneumonia, and Mycoplasma pneumonia. However, samples showed the presence of *Moraxella*, *Streptococcus*, *Nocardia*, *Lactobacilli*, and *Legionella*. In aggravated state samples *Haemophilus influenza*, *Staphylococcus aureus*, *Staphylococcus pneumonia*, *E. coli*, *Lactobacillus*, *Bifidobacteria*, and *Nocardia* were observed. **Conclusion:** The current findings suggest that there is a unique relationship between bacterial pathogens and the recurrence of the severity of symptoms in COPD. To understand the diversity of atypical and typical microbiota it is important to utilize sensitive advanced techniques for routine investigations of the COPD population.

Keywords: Chronic Obstructive Pulmonary Disease, COPD, Haemophilus, Moraxella, Nocardia, Legionella, Bifidobacter

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INTRODUCTION

Chronic obstructive pulmonary disease (COPD)[1] is a chronic respiratory disease characterized by progressive, debilitating respiratory conditions, including emphysema and chronic bronchitis, characterized primarily by dyspnoea, lung airflow limitations, cough, sputum production, and other symptoms.

Chronic obstructive pulmonary disease is a major cause behind a heavy health and economic burden around the world.[2] According to the Global Burden of Diseases Study (GBD) 2017 reports[3], COPD resulted in 3.2 million deaths. It accounted for 81.6 million disability-adjusted life years (DALYs).[3]

Exacerbation of chronic obstructive pulmonary disease is mainly due to increased respiratory tract inflammation. Patients suffering from acute COPD exacerbation have impaired health status, a higher risk of lower airway bacterial colonization, and accelerated lung function decline.[4] It was observed that more than half of COPD patients were infective, and bacteria were isolated from 70% of their samples.[5]

Among various bacterial species involved in infectious exacerbations, the most common bacteria are *Haemophilus influenza*, followed by *Streptococcus pneumoniae* and *Moraxella catarrhalis*.[6] However, atypical pathogens like *Mycoplasma pneumonia*, *Chlamydia pneumonia*, and *Legionella pneumophila* may not play important role in acute exacerbation of COPD.[7]

Conventional methods have proved to be instrumental in identifying that bacterial pathogens play a pivotal role in the recurrence of COPD symptoms. Sputum culture has identified bacteria, which account for 30–50% of COPD exacerbations.[8] However, due to its limited ability, we have also used sensitive real-time Polymerase Chain Reaction (PCR) assays. PCR can detect tiny fragments and small fragments of nucleic acids of all pathogens, and it is not dependent upon the capability of the target microbe.

This study is based on analysis of respiratory tract samples by comprehensive real-time polymerase chain reaction to detect bacterial species together with conventional methods, including sputum culture. It focuses on the evidence that the recurrence of the severity of symptoms is due to the presence of bacterial etiological agents.

METHODOLOGY

This retrospective, randomized double-blind study was aimed to analyze 120 clinically diagnosed COPD patients enlisted from tertiary care hospitals of Karachi having age ≥40 from 21 Feb to 17 Aug 2018. Sample of Sputum was collected from the outpatient department and from inpatients who were admitted in wards due to severity of illness at Sindh Government Lyari General Hospital. Written and informed consent from COPD patients was taken.

Data was collected from each patient and recorded on a pre-tested questionnaire. The questionnaire included duration of hospitalization, smoking habit, fever, antibiotic usage, and duration of symptoms before admission. Variables included for the study were age, sex, signs, and symptoms of the patient.

Subjects were divided into two equal groups. Group-A consisted of 60 randomly selected COPD subjects with acute severity of symptoms. The sputum samples of this group were marked as aggravated state sputum. Group-B consisted of 60 COPD subjects with no acute severity of symptoms their sputum samples were marked as stable state sputum.

After rinsing their mouths, the COPD patients were instructed to collect early-morning, deeply coughed sputum samples into a sterile wide-mouth container with a screw cap. All the sputum samples were incubated at 37 °C for 15 min with a volume of 0.1% dithiothreitol was added. Then each sample was divided into two equal portions: one part was utilized for gram staining and culturing sputum samples, while the other was subjected to two different PCR techniques. Each portion had $<10^5$ epithelial cells/mL which is equal to <1 epithelial cell per high-power field.

The collected sample was subjected for culture and DNA extraction within 4–5 hours. Two hundred (200) µL sputum sample was treated using NucliSens® easyMAG™ (bioMérieux, USA) according to the manufacturer's instructions. Five (5) µL sputum samples were utilized as a PCR template. DNA was stored at -20 °C until PCR was done.

For identification of *E. coli*, *Lactobacillus*, *Bifidobacteria*, *Legionella*, and *Nocardia* two different assays were utilized directed on explicit zones of 5S *r*RNA and macrophage infectivity potentiator (*mip*) gene. PCR-probe assay for *E. coli*, *Lactobacillus*, *Bifidobacteria*, *Nocardia*, and *Legionella*, are based on the primers, and Leg5S detected in real-time using a TaqMan probe, Netherlands. For identification of *Haemophilus influenza*, *Staphylococcus aureus*, and *Staphylococcus pneumonia* an assay was performed using 2 and 4 outer membrane protein, variable nucleotide sequence. This PCR was based on the sequences of the *mip* gene, P1 adhesion gene assay. Sixty-six 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′. Fluorescent labelled 6-carboxyfluorescein Taqman probe 5′-TCCCCC GTT GAA AAA GTG AGT GGG T-3′ was used for Real-time PCR.

All the samples were processed for DNA extraction using both Qiagen and microLYSIS techniques. This was done to detect every possibly available bacterial DNA present in the samples and avoid missing microbiota.

This research was conducted using open epic software. Statistical analysis was done using SPSS-20. The data of categorical variables were presented as counts and percentages. Descriptive frequencies were used for analyzing all categorical data.

All clinically diagnosed cases of COPD and acutely exacerbated COPD formed the subject of the study group. Patients with previous diagnosis of any active lung disease, on treatment with oral steroids, pulmonary disease of allergic origin, and patients on antibiotics before hospitalization were not included in the study.

RESULTS

One hundred and twenty (120) samples were collected from COPD patients from the outpatient and inpatient Departments of Sindh Government Lyari General Hospital. Diagnosed patients of age ≥40 years with COPD were divided into two groups based on the severity of symptoms. Group-A included 60 randomly selected COPD subjects with acute severity of symptoms. Group-B included 60 randomly selected COPD subjects with no acute severity of symptoms (Table-1).

Table-2 compares bacterial detection capability of culture assay and PCR technique. The detection capacity of simple culture techniques was less than that of PCR. In Group-A (Aggravated State Sputum) the efficacy of culture techniques were 18/60 (30%) in comparison with PCR having a detection capability of 45/60 (75%). In Group-B (Stable State Sputum) the detection capacity of the culture technique was 27/60 (45%) and PCR was able to detect 40/60 (66%) cases. On comparing results of both groups the PCR method had better detection capacity as compared to conventional culture techniques.

DNA was extracted in triplicates, from each pure culture using the Qiagen and the microLysis techniques. 16S qPCR assays were performed to enumerate the genome/ml readings from the extracted DNA. CFU/ml counts were performed. Results depicted that microLYSIS technique towards the detection of atypical bacteria is slightly better than Qiagen. However, the study was unable to identify any major differences in detection of bacteria between both techniques. (Table-3).

Figure-1 represents the gender-wise group distribution on its X-axis, while it shows the log (CFU/ml and Genome/ml) on its Y-axis. The different colour bars show the type of bacteria being identified in each group.

In group A the male gender showed a high presence of *Nocardia* along with *Homophiles*, *E. coli*, *Moraxella*, *Bifidobacteria*, and *Lactobacillus* (highest to lowest order). In group A the female gender showed *Nocardia* along with, *Moraxella*, *Homophiles*, *E. coli*, *Lactobacillus*, *Streptococcus*, and *Bifidobacteria* (highest to lowest order).

In group B the male gender showed a high presence of streptococcus along with *Homophiles*, *Moraxella*, *E. coli*, *Nocardia*, *Bifidobacteria*, and *Lactobacillus* (highest to lowest order). In group B the female gender showed *Homophiles* in highest percentage along with *E. coli*, *Moraxella*, *Streptococcus*, *Nocardia*, *Bifidobacteria*, and *Lactobacillus* (highest to lowest order).

Table-1: General characteristics of study population

Gender	Age Group	Average Age	Occupation	Residence	Past personal habits/ Occupational history	Therapeutic History
Group-A Aggravated State Sputum (n=60)	**Males** (n=35)	55	Factory workers/ Skilled Labourers	Karachi	Smoker+Occupational	Non Compliant/Symptomatic treatment
	Females (n=25)	50	House wives	Karachi	Gutka+Huqqa+Biomass Fuel stove	Non Compliant/Symptomatic treatment
Group-B Stable State Sputum (n=60)	**Males** (n=31)	50	Factory workers/ Skilled Labourers	Karachi	Gutka+Huqqa	On regular treatment+follow up
	Females (n=29)	42	House wives	Karachi	Gutka+Huqqa+Biomass Fuel stove	On regular treatment+follow up

Table-2: Comparison between Conventional microbiological results and Molecular microbiological results

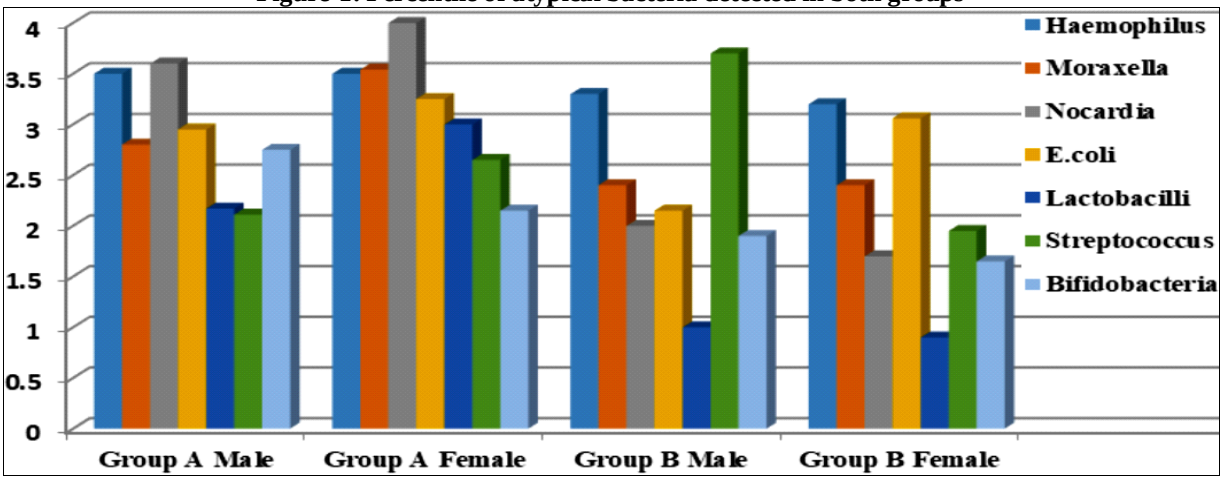
Groups	CULTURE (n=120)	PCR (n=120)
Group-A Aggravated State Sputum (n=60)	18/60 (30%)	45/60 (75%)
Group-B Stable state sputum (n=60)	27/60 (45%)	40/60 (66%)

Table-3: Comparison between QIAGEN and Microlysis DNA bacterial detection

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

Figure-1: Percentile of atypical bacteria detected in both groups



X-axis: Group Division gender-wise, Y-axis: log (CFU/ml and Genome/ml)

DISCUSSION

Chronic obstructive pulmonary disease exacerbation is one of the major causes of morbidity in chronic respiratory diseases patients.[3] It has a strong economic burden and also strongly influences health-related quality of life (HRQL).[9] This study was conducted to delineate the most common typical and atypical bacterial agents involved in COPD exacerbations.

Previous reports have shown the association between acute COPD exacerbation and the presence of bacterial pathogens by using only a single procedure, either conventional microbiological assays (sputum culture) or Polymerase chain reaction (PCR), while the use of a combination of techniques was extremely occasional. The current study was conducted using the combination of both the culture and the PCR assays.

When conventional culture techniques were compared with real-time PCR it was observed that the accuracy of real-time PCR was slightly superior to the conventional techniques. These findings were quite similar to the findings of Shimizu K *et al*[8]. Only a few differences were observed in the capacity of both techniques.

Culturing sputum samples is considered the first-line investigation, for exploring the pathogenesis of exacerbations of COPD. They are cost-effective and provide the pathogens to be studied further. However, when used alone, sputum culture technique has many limitations.[10] Researchers like Aydemir *et al*[11], Halder *et al*[12], and Perotin *et al*[13] had also used PCR methods along with culture techniques to compensate for the shortcomings of conventional methods.

Talking about PCR assays, on comparing its different DNA extraction techniques, the most commonly used techniques were Qiagen and microLYSIS. It was observed that both techniques have almost the same efficacy in the identification of microbiota. However, the capacity of the microLYSIS technique towards the detection of atypical bacteria is slightly better than Qiagen. The focus of this study was to examine the predominant bacterial (typical or/and atypical) pathogens associated with exacerbation of COPD.

It was an astonishing observation that *Nocardia* became the predominant pathogen log/ml in both sexes of the group suffering from acute exacerbation of symptoms. It is one of the most transmitted bacteria by inhalation or aspiration.[14] It particularly affected immunocompromised patients. In recent research by Garcia-Bellmunt L *et al*[15] an increase in *Nocardia* infection cases has been reported among patients with COPD. Though the factors behinds its association with exacerbation of COPD is unknown, it was observed that corticosteroid therapy was prevalent amongst individuals diagnosed with *Nocardia* infection.[16]

Another important finding in Group-A is *Haemophilus*, *Moraxella*, and *E. coli* in high percentage log/ml in the acute exacerbation group. According to Sethi S[17], three predominant bacterial species were isolated from patients experiencing an exacerbation of COPD. Those three pathogens were *Haemophilus influenzae*, *Moraxella catarrhalis*, and *Streptococcus pneumoniae*. These three species were also among the most common pathogens responsible for other respiratory mucosal infections. *H. Influenzae* has also been reported to be the most common isolate.[18]

Monso *et al*[19] concluded that the potential bacterial pathogens cultured from sputum during COPD exacerbations were less frequently cultured during the period of clinical stability of the COPD patient. In the current study, it is well established finding that in the acute exacerbation phase bacteria for the respiratory tract such as *Moraxella*, *E. coli*, and *Lactobacillus* were identified in the group with recurrence of acute exacerbation of symptoms in both sexes. *Lactobacilli* and *Bifidobacteria* were also identified in more than normal percentage log/ml in the acute exacerbation group. Research by Sethi *et al* identified differences in culture densities of *Haemophilus influenza*, *Moraxella catarrhalis*, and *Streptococcus pneumonia* in sputum collected during acute exacerbations compared to samples during clinical stability.[20] *Lactobacilli* association with acute exacerbation of COPD was also observed during the research. This finding is supported by Sze MA *et al*, who also detected an increase in the *Lactobacillus* genus (i.e., *Firmicutes* community) in severe COPD exacerbation.[21]

The relationship between COPD exacerbation and acute bacterial infection of the respiratory airway is still debatable. However, these findings indicate the probable positive association of these pathogens with recurrent acute exacerbations of symptoms of COPD patients in our subjects.

It has been observed that one of the major issues in the non-uniformity of data is due to regional variation[22,23] and segregated methodology for identification of pathogens. This lack of standardization has lead to the variation of published data concerning COPD. The mechanism which can be postulated is the use of random medication and antibiotics along with the non-quantified dosage of steroid-based drugs. Guidelines for antibiotic therapy for COPD patients have been published by many researchers like Llor C *et al*[24], but these cannot be applied on a worldwide basis due to geographical variations in antibiotic sensitivity and pathogen types. However, judicious use of antibiotics is crucial to avoid the emergence of multidrug-resistant bacteria.

CONCLUSION

The PCR assays showed more sensitivity toward detecting bacteria in sputum samples than the conventional assays. *Nocardia* emerged as a major pathogen, irrespective of patient's gender. Moreover, the bacteria like *Bifidobacteria*, *Moraxella*, *E. coli*, and *Lactobacillus* were identified in the group with the recurrence of acute exacerbation of symptoms. *Haemophilus* was among some majorly found bacteria. Bacteria are the major cause of acute COPD exacerbation. Regional variation and the difference in methodology for identification of pathogens were some of the main reasons behind the non-uniformity of data. The researchers should increase usage of novel molecular techniques along with other conventional detecting methods for better detection of bacterial communities.

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ZL: Critical analysis and corrections
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AMS: Concept and review

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