Original Article

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Internal Medicine Section

Saliva as a Non Invasive Specimen for Assessment of Chronic Obstructive Pulmonary Disease: A Cross-sectional Study

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ABSTRACT

Introduction: Chronic Obstructive Pulmonary Disease (COPD) is a leading cause of morbidity and mortality worldwide. Impaired mucociliary clearance due to altered respiratory physiology in COPD presents an exceptional opportunity for bacterial proliferation. Sampling the respiratory tract using sputum or Bronchoalveolar Lavage (BAL) can be labourious and inconvenient, particularly in chronically debilitated patients. Saliva offers an interesting and non invasive method for assessing COPD patients and preventing exacerbations.

Aim: To use saliva to analyse the association between the frequency of positive Potentially Pathological Bacterial Isolates (PPBI) and COPD exacerbations in relation to the frequency of exacerbations and the severity of the disease.

Materials and Methods: This cross-sectional study was conducted over a period of one year (July 2022 to June 2023) among COPD patients attending the outpatient department at the Department of Respiratory Medicine, GMERS Medical College, Gotri, Vadodara, Gujarat, India. A total of 60 patients with COPD, diagnosed according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) 2021 guidelines, were included. The patients were categorised based on the severity of airflow limitation, GOLD "ABCD" assessment tool, and number of exacerbations. Salivary samples were collected and subjected to microbiological analysis using laboratory conventional culture techniques. Analysis of Variance (ANOVA) and t-tests were applied.

Results: The mean age was 64±5.1 years. *S. pneumoniae* and *H. influenza* were common bacterial findings in all stages of COPD, while *E. coli* and *A. baumannii* were isolated in GOLD Group D patients. Disease severity also showed a significant association with oral bacterial composition (p=0.010) and the frequency of exacerbations (p=0.03).

Conclusion: The current study demonstrates an association between oral bacteria and COPD, especially in patients with severe symptoms (GOLD Group D). Additionally, patients with repeated exacerbations exhibited a different oral bacterial composition, thus supporting the use of saliva as a non invasive specimen for assessing heterogeneous diseases like COPD and designing an empiric antibiotic regimen for those PPBI.

Keywords: Bronchoalveolar lavage, Global initiative for obstructive lung disease, Pathologically positive bacterial isolates

INTRODUCTION

Chronic Obstructive Pulmonary Disease (COPD) is a preventable and treatable disorder characterised by progressive respiratory symptoms caused by airway obstruction and airway/alveolar abnormalities resulting from exposure to harmful particles. Pathophysiological respiratory changes hinder mucociliary clearance, creating a favourable environment for bacterial proliferation. Studies conducted on BAL and sputum samples from COPD patients have revealed distinct airway microflora compared to healthy individuals [1]. A positive correlation has been reported between the disease's severity and the microflora composition, with more severe patients exhibiting enrichment in *Proteobacteria* (50%), *Haemophilus* (25%), and *Moraxella* (3%) [1,2]. The composition of oral microflora could potentially serve as a valuable biomarker for evaluating COPD severity [3], although evidence supporting this assertion is currently lacking.

Studies [4,5] use bronchial secretions to evaluate the microbiological causes of COPD exacerbation. For instance, a study by Rosell A et al., showed that a quarter of COPD patients are colonised by PPBI during stable periods [4]. Exacerbations of COPD are linked to the overgrowth of these PPBs and the appearance of *Pseudomonas aeruginosa*, which is associated with exacerbation symptoms independently of bacterial load. Furthermore, a study by Garcia-Nunez M et al., demonstrated a clear relationship between the severity of airway obstruction and decreasing bacterial community diversity. This implies that in patients with more severe obstruction, bacterial community diversity decreases, increasing the likelihood of finding individual PPBI [5].

While these studies offer insights into the relationship between disease severity and microbial composition, a limitation of these studies is the use of induced sputum or BAL samples. Sputum induction or BAL sampling are relatively invasive and resource-intensive procedures that require experienced healthcare personnel and expensive instruments. Additionally, performing bronchoscopy in COPD patients is associated with a significantly higher risk of pneumonia, respiratory failure, and bleeding [6]. Sputum induction processing methods, though semi-invasive, are labourious, especially when sampling debilitated patients such as those with neurological deficits or postoperative patients [7,8].

Saliva, a non invasive specimen from the upper respiratory tract, can be easily obtained from patients in an outpatient setting as well as from chronically debilitated patients. This is based on the assumption that the upper and lower respiratory tract microbiota exhibit topological continuity and that oral bacteria mainly colonise the lower respiratory tract through microaspiration [9]. Consequently, the microbiota of the lower respiratory tract has lower diversity compared to the microflora of the upper respiratory tract [10].

Previous studies using saliva to assess COPD, such as the work by Melo-Dias S et al., have recognised an association between oral bacterial composition and COPD [11]. The current study aimed to establish a link between the frequency of positive PPBI and the

evaluation of COPD patients during exacerbations concerning the frequency of exacerbations and disease severity. These PPBIs are identified and obtained using conventional culture methods and biochemical tests.

MATERIALS AND METHODS

This cross-sectional study was conducted over a period of one year (July 2022 to June 2023) among COPD patients attending the outpatient department at the Department of Respiratory Medicine, GMERS Medical College, Gotri, Vadodara, Gujarat, India. The study included 60 patients with COPD diagnosed according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) 2021 guidelines [12]. The study was approved by the Institutional Human Ethics Committee (IHEC) (Letter No: IHEC/22/OUT/SRUG016), and informed consent was obtained from all study participants.

Inclusion criteria:

- Patients diagnosed with COPD according to the GOLD 2021 guidelines [12].
- Age between 35 and 90 years.
- Patients of both sexes.

Exclusion criteria:

- Patients with an alternate or co-existent diagnosis, such as bronchial asthma, interstitial lung disease, bronchiectasis, or pulmonary fibrosis.
- Patients with cardiac diseases like coronary artery disease, ischaemic heart disease, or valvular heart disease.
- Patients with Human Immunodeficiency Virus (HIV), organ transplants, connective tissue disorders, or altered cognitive function.

Sample size calculation: Eligible patients attending the department of respiratory medicine were surveyed using convenience sampling until the required sample size was achieved. The formula for calculating sample size was, $n=z^2p(1-p)/d^2$, where: Z=1.96 indicates a significance level of 0.05 and a confidence level of 0.95 or 95%. p=7%, COPD prevalence is estimated at 7% [13], and d=0.065, a margin of error or 6.5% absolute precision. The calculated sample size was 60 based on these parameters.

Study Procedure

A detailed history and physical examination was done on all patients. All patients were subjected to salivary examination.

Saliva collection procedure: Patients were instructed not to eat, drink, or smoke 30 minutes before donating saliva. They were advised to avoid brushing or gargling with mouthwash on the morning of sample collection. All passive saliva collection appointments were scheduled between eight and ten in the morning. Before collection, the patient's parotid area was gently massaged with the mouth closed to collect a more viscous sample. Then, the patients were asked to spit out the saliva into the universal container. All the saliva samples underwent the following culture methods for identifying organisms.

Laboratory analysis: The salivary sample was then subjected to the following microbiological analysis:

a. **Cultivation procedure:** Chocolate agar, blood agar, nutrient agar, and McConkey agar were used as the culture media. The sample was inserted into a culture dish containing a medium that promotes bacterial growth. The dish was then placed in a bacterial incubator at 37°C. Upon obtaining positive culture results, the exact type of bacteria was identified by performing microscopy, colony morphology, or biochemical tests for bacterial growth. In the case of mixed growth, subcultivation was performed to support the growth of isolates.

b. **Stain test procedure:** The cultivated bacterial colonies were then placed on a glass slide to be stained with appropriate staining dyes. The slides were labelled positive if bacteria were observed under a microscope.

c. **Biochemical test procedure:** To identify the organism, bacteria were first inoculated into a series of subcultures. Organisms were then identified using indicators and products of metabolism within the medium.

d. **Serological test procedure:** Enzyme-linked Immunosorbent Assay (ELISA) was used to measure the antibodies against non encapsulated *Haemophilus influenza* and *Moraxella (Branhamella) catarrhalis*. An agglutination technique against *H. influenzae* serogroup A and serogroup C antigens was also performed.

Spirometry was performed on all patients who complied with the American Thoracic Society/European Respiratory Society 2022 recommendations [14]. All patients with COPD were assessed and classified according to the Modified Medical Research Council (mMRC) scale for dyspnoea [15], the COPD "ABCD" assessment tool [16], GOLD Stages of severity of airflow limitation [16], and the Number of COPD Exacerbations (NoEs).

The mMRC scale is a self-rating tool used to measure the degree of disability that breathlessness poses in day-to-day activities on a scale from 0 to 4: 0, no breathlessness except during strenuous exercise; 1, shortness of breath when hurrying on level ground or walking up a slight hill; 2, walks slower than people of the same age on level ground due to breathlessness or has to stop to catch breath when walking at their own pace on level ground; 3, stops for breath after walking approximately 100 m or after a few minutes on level ground; and 4, too breathless to leave the house, or breathless when dressing or undressing [15].

The severity of COPD can be assessed through spirometry by measuring the extent of airway obstruction or limitation [16]. Spirometry involves performing a forced expiratory maneuver once a patient has inhaled to total lung capacity. The FEV1 refers to the Forced Expiratory Volume in 1 second (FEV1), which is the volume of air exhaled during the first second of this maneuver. The total volume of air emitted during the maneuver is the Forced Vital Capacity (FVC). Airflow obstruction is characterised by a decrease in the ratio of FEV1 to FVC. Accordingly, COPD can be categorised as mild, moderate, severe, and very severe [Table/Fig-1].

Stage	Condition	Characteristics
I	Mild COPD	maneuo1 ≥80% predicted
II	Moderate COPD	50% ≤FEV1 <80% predicted
III	Severe COPD	30% ≤FEV1 <50% predicted
IV	Very Severe COPD	FEV1 <30% predicted

[Table/Fig-1]: Classification of COPD severity according to severity of airflow limitation.
FVC: Forced vital capacity; FEV1: Forced expiratory volume in 1 second

The COPD GOLD 2021 guidelines use the COPD "ABCD" assessment tool [16], which assesses symptoms, breathlessness according to the mMRC scale, spirometry classification of airflow limitation, and the number of exacerbations to classify patients into the following groups:

- **Group A** (low risk/less symptoms): 1 or fewer exacerbations per year with no hospitalisation, mMRC 0-1, or COPD Assessment Test (CAT) score less than 10
- **Group B** (low risk/more symptoms): 1 or fewer exacerbations per year with no hospitalisation, mMRC 2 or higher, or CAT Score 10 or higher
- **Group-C** (high risk/less symptoms): 2 or more exacerbations per year with 1 or more exacerbation requiring hospitalisation, mMRC 0-1, or CAT Score less than 10
- **Group D** (high risk/more symptoms): 2 or more exacerbations per year with 1 or more exacerbation requiring hospitalisation, mMRC 2 or higher, or CAT Score 10 or higher

STATISTICAL ANALYSIS

Microsoft excel (2021) and Statistical Package for Social Sciences (SPSS) version 26.0 software were used to evaluate all the data. Frequencies, percentages, and means, as appropriate, were used to characterise the data. Chi-square tests, ANOVA tests, and linear regression were applied for the effective interpretation of the results. A p-value lower than 0.05 was considered significant.

RESULTS

The study was conducted on a total of 60 patients. Most of them, 51 (85%), were males, while the remaining 9 (15%) were females [Table/Fig-2]. The ratio of female to male patients was 1:5.6. The majority of patients were aged 60 to 69 years, which is 21 (35%) patients [Table/Fig-2]. The maximum age was 85 years, while the minimum age was 38 years. The mean age was 64±5.1 years.

Characteristics	Frequency (%)	
Gender	Male	51 (85)
	Female	9 (15)
Age (years)	30-39	2 (3.3)
	40-49	14 (23.3)
	50-59	15 (25)
	60-69	21 (35)
	70-79	7 (11.6)
	80-89	1 (1.7)
Symptoms	Cough	51 (85)
	Dyspnoea	47 (78.3)
	Chest pain	24 (40)
	Fever	13 (21.6)
	Others*	4 (6.6)
mMRC grading of dyspnoea	Grade 0	0
	Grade 1	23 (38.3)
	Grade 2	7 (11.6)
	Grade 3	14 (23.3)
	Grade 4	16 (26.6)
GOLD staging of airflow limitation	Stage I	16 (26.6)
	Stage II	9 (15)
	Stage III	15 (25)
	Stage IV	20 (33.3)
COPD "ABCD" assessment tool	A	11 (18.3)
	B	12 (20)
	C	14 (23.3)
	D	23 (38.3)

[Table/Fig-2]: Distribution according to age, sex, symptoms, mMRC grading for dyspnoea, GOLD staging of airflow limitation and COPD "ABCD" assessment tool. *(Other symptoms include body ache, headache, and anorexia)

Cough was the most common symptom in respiratory illness that brought the patients to the physician and was present in 51 patients (85%). Most of the patients had a cough associated with a mild to moderate amount of expectoration. Dyspnoea was the second most common symptom present in 47 patients (78.3%) [Table/Fig-2]. About 49 (81.6%) males had a smoking history, out of which 33 males (67.3%) were current smokers. Biomass fuel exposure was present in 8 patients (13.3%), all women.

When assessing the severity of airflow limitation in COPD, 20 (33.3%) patients belonged to Stage-IV (very severe), 15 (25%) patients to Stage-III (severe) COPD, while 9 (15%) patients had Stage-II (moderate) COPD [Table/Fig-2]. According to the COPD "ABCD" assessment tool, 14 (23.3%) patients belonged to Group-C, 12 (20%) patients to Group B, and 11 (18.3%) patients to Group A [Table/Fig-2].

In the present study, *S. pneumonia* and *H. influenza* were common bacterial findings in all stages of COPD, while *E. coli* and *A. baumannii* were isolated in GOLD D patients [Table/Fig-3]. When applying linear regression with ANOVA to test the association between oral bacterial findings and the COPD "ABCD" assessment tool, oral bacterial findings were able to predict severely symptomatic COPD patients (GOLD group D) with a variance of 29.6% in GOLD stages (p-value <0.001) [Table/Fig-4].

Name of the bacteria	COPD "ABCD" assessment tool patient groups				Total
	A	B	C	D	
Normal flora	5	3	3	1	12
Streptococcus pneumoniae	2	5	2	4	13
Haemophilus Influenza	3	1	1	2	7
Moraxella catarrhalis	1	2	2	0	5
Klebsiella pneumoniae	0	0	3	5	8
Pseudomonas aeruginosa	0	1	2	4	7
Escherichia coli	0	0	0	3	3
Staphylococcus aureus	0	0	1	3	4
Acinetobacter baumannii	0	0	0	1	1
Total	11	12	14	23	60

[Table/Fig-3]: Distribution of bacterial finding in different groups of patients.

Regression and weights	Beta coefficient	R2	F	p-value	Association
Bacterial findings and GOLD grouping	0.546	0.296	24.61	<0.001	Yes

[Table/Fig-4]: Linear regressive analysis between oral bacterial findings and GOLD grouping.

There was a significant association, at the 5% significance level, between the severity of GOLD staging and the isolation rate of PPBI (x2=8.182, df=3, p=0.042) [Table/Fig-5]. Also, there was a significant association (p<0.05) when ANOVA was applied between the frequency of exacerbations and the isolation of PPBI (X2=4.2714, df=1, p=0.038795) [Table/Fig-6]. Disease severity also showed an association with oral bacterial composition at a 5% significance level (X2=20.008, df=8, p=0.010) as shown in [Table/Fig-7].

GOLD "ABCD" tool patient groups	PPB isolates		Total
	Negative	Positive	
A	5	6	11
B	3	9	12
C	3	11	14
D	1	22	23

[Table/Fig-5]: Association (p=0.042) between GOLD grouping and isolation rate of PPBI.

Variables	Number of exacerbations		Total
	0-1	>=2	
PPB isolates+	20	28	48
PPB isolates -	9	3	12
Total	29	31	60

[Table/Fig-6]: Association between isolation rate of PPB Isolates (PPBI) and number of exacerbations.

DISCUSSION

The present study was designed to investigate the association between oral bacterial composition and GOLD stage. The present study demonstrates that oral bacterial findings were able to predict severely symptomatic COPD patients, i.e., GOLD Group D. This can help identify patients who are at risk of frequent exacerbations and thus help in their management. The study also showed a significant association between GOLD stages and the rate of positive PPBI. The

Bacteria	GOLD stages of severity of airflow limitation in COPD				Total
	Mild GOLD I	Moderate GOLD II	Severe GOLD III	Very severe GOLD IV	
Normal flora	4	4	3	1	12
Streptococcus pneumoniae	4	2	3	4	13
H. influenza	3	1	2	1	7
Moraxella catarahhalis	2	1	2	0	5
K.Pneumoniae	2	1	1	4	8
P. aeruginosa	1	0	2	4	7
E. coli	0	0	1	2	3
S. aureus	0	0	1	3	4
A. Baumani	0	0	0	1	1
Total 60					

[Table/Fig-7]: Association between GOLD classification of airflow limitation and oral bacterial composition.

present study showed that GOLD Group D patients have a higher chance of finding PPBI. Thus, the present study demonstrated that the more at-risk and symptomatic the patient, the less diverse the community of bacterial diversity, and the greater the chance of finding PPBI, making it easier to investigate the causes and stabilise the patient.

Melo-Dias S et al., reported similar results in a study where moderate patients (GOLD 1 and 2) had significantly different oral bacterial compositions when compared with severe patients (GOLD 3 and 4) [11]. In the present study, *S. pneumoniae* and *H. influenzae* were common bacterial findings in all stages of COPD, while *A. baumannii* was isolated in GOLD D patients. The present study also showed a significant association between oral bacterial composition and disease severity in people with COPD. This means that high-risk, more symptomatic patients can be significantly predicted with the isolation of Pseudomonas, *E. coli*, and *A. baumannii*. These results were similar to a study conducted by Rosell A et al., where bronchoscopy was used for sampling lower respiratory tract specimens. They concluded that a higher microbial load is associated with COPD exacerbations, especially with a predominance of *H. influenzae* and *P. aeruginosa* [4]. Another study from Beasley V et al., used the induced sputum technique to find microbiological determinants of COPD exacerbations. The exacerbation samples were found to have higher concentrations of H. influenzae in the study [17]. While previous research has used invasive or semi-invasive techniques to assess microbiota findings in people with COPD [4,5], the present study provides new insight into non invasive and patient-friendly sampling such as saliva.

The presence of dynamic host defenses prevents infection from developing in healthy individuals, despite the constant inhalation of bacteria. Host defenses include both anatomical and physiological factors. Airway macrophages, secretory Immunoglobulin A (IgA), antimicrobial peptides, as well as a compact epithelial lining and mucociliary clearance, play a pivotal role in preventing disease in healthy individuals [18]. The presence of a bacterial infection in the lower respiratory tract indicates that the host's lung defenses are compromised. Cigarette smoking is a significant risk factor in damaging the mechanical barriers in the lungs, which can support the development of infection [19]. Infections in COPD may also be caused by genetically acquired defects in the function of airway immune cells, such as neutrophils and alveolar macrophages. In smokers and COPD patients, the airways have an increased level of macrophages and neutrophils. The ability of macrophages to phagocytose microorganisms is impaired in COPD, as shown by various studies [19,20]. Decreased levels of toll-like receptor TLR2 on the cell surface of immune cells in patients with COPD cause defective macrophage function in COPD [21]. The binding and uptake of Gram-positive and Gram-negative bacteria by macrophages is mediated by Macrophage Receptor with Collagen Structure (MARCO), a class A scavenger receptor. The expression of this receptor is diminished by cigarette smoking [22].

The frequency of exacerbations and the rate of positive PPBI were found to have a significant association in the current study. A study by Patel IS et al., reports similar findings but also explains that lower bacterial colonisation in stable COPD modulates the nature and frequency of exacerbations [23].

Determining the relationship between oral bacterial composition and clinical features, although not significant, was found to predict GOLD stages, i.e., risk assessment and symptoms. This can prove to be valuable in designing a correct antibiotic regimen, which will help reduce the morbidity and mortality of the disease. The results of the present study confirm previously reported findings that used BAL and induced sputum samples [4,5]. The use of a non invasive technique is what makes the present study unique and different from previously published studies. These results should be considered when evaluating and treating COPD in debilitated patients with a neurological deficit or in patients at risk of laryngospasm, bronchospasm, etc. The current study shows saliva has the potential to be a promising biomarker for evaluating COPD patients.

Limitation(s)

Using the 16S ribosomal Ribonucleic Acid (rRNA) gene amplification method to profile oral bacteria can provide further insights into the oral composition of the patients. However, this was not done in the present study.

CONCLUSION(S)

The present study suggests an association between symptomatic COPD patients (i.e., GOLD Group D) and oral bacterial morphology. Additionally, the study shows a significant association between the frequency of exacerbations and the rate of PPBI. Saliva can prove to be a useful tool for assessing COPD patients, particularly chronically debilitated patients, thus avoiding the side-effects of semi-invasive and invasive sampling methods. This may help in designing an empiric antibiotic regimen for those with PPBI.

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