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Air Way Bacterial Colonization in COPD Patients at Stable and the Exacerbated States

Wedad M. Abdelraheem[1]*, Ebtisam S. Mohamed[1], Elham A. Abdelghany[2], Rasha M. Emam[2] and Manar S. Mohamed[3]

[1] *Department of Medical Microbiology and Immunology, Minia University, Minia, Egypt*

[2] *Department of Chest Diseases and Tuberculosis, Minia University, Minia, Egypt*

[3] *Department of Internal Medicine, Deraya University, Minia, Egypt*

**Corresponding e-mail: altaqwa.2012@yahoo.com*

ABSTRACT

Objectives: *The aim of this study was to investigate the prevalence and load of airway bacteria in chronic obstructive pulmonary disease (COPD) patients and to detect the antibiotic susceptibility pattern of the isolated strains.* ***Methods:*** *The current study included 50 COPD patients. Paired sputum samples were taken from all patients, one at the exacerbation state and the other at the stable state. Identification of bacterial species was done using standard microbiological techniques and PCR.* ***Results:*** *There were 41 samples out of 50 (82%) positive for bacteria at exacerbation compared with 12 samples out of 50 (24%) positive for bacteria (either by culture or PCR or both) at stable state samples (p=0.001). The most commonly detected species was H. influenza and S. pneumoniae. There were a total of 27 bacterial species detected by PCR not detected by culture and only 4 bacterial species detected by culture not detected by PCR (p=0.001). Isolated strains show a high resistance pattern to the tested antibiotics and multi drug resistant strains were detected.* ***Conclusions:*** *This study has shown that airway bacterial prevalence and load increase at COPD exacerbations, and there is a significant relationship between lower airway bacterial colonization and COPD.*

Keywords: COPD, Stable, Exacerbation, Culture, PCR

INTRODUCTION

COPD is a progressive inflammatory disorder characterized by airflow limitation due to a mixture of airway disease, chronic airway inflammation, parenchymal destruction, and increased airway responsiveness [1]. COPD is associated with a considerable rate of morbidity and mortality and has been projected to be the fourth highest cause of death by 2030 [2].

The chronic course of COPD is often complicated by acute exacerbations, mainly due to increased inflammation [1]. Acute exacerbations of COPD are episodes which are characterized by worsening of respiratory symptoms associated with physiological deterioration [3]. Patients prone to frequent acute exacerbation have impaired health status, reduced physical activity, increased airway bacterial colonization, and progressive lung function decline [4].

These events are a major cause of morbidity and mortality, and COPD exacerbations are a leading cause of hospital admissions [3]. Thus, the management of exacerbations with accurate diagnosis and effective treatment should be a major goal in COPD patients [4]. The majority of COPD exacerbations are caused by infections of the respiratory system [5]. A key characteristic of airway inflammation in COPD is the persistent presence of bacteria in the lower respiratory tract. The most commonly isolated bacteria in the lower respiratory tract of COPD patients were *H. influenzae, S. pneumonia, M. catarrhalis* and *P. aeruginosa* [6]. The majority of studies of bacterial prevalence and load in COPD patients have been based on culture, but there is little on PCR comparison with culture.

The aim of this study was to investigate the prevalence and load of airway bacteria in COPD patients at the stable and exacerbation states and to determine the antibiotic susceptibility and resistance patterns of the isolated strains. We also compared PCR with routine microbiological culture results.

PATIENTS AND METHODS

Study Population

All patients diagnosed previously for COPD and admitted with acute exacerbation (AECOPD) in the Department of Chest Diseases, Minia University Hospital, during the period between September 2016 and June 2018, were candidates for inclusion in the study. COPD diagnosis was based on medical history, clinical presentation, and pulmonary function tests. Exclusion criteria were a diagnosis of another acute respiratory condition (e.g. pneumonia, pneumothorax, or pulmonary embolism), history of respiratory disorders other than COPD (e.g. asthma, bronchiectasis, pulmonary fibrosis, and pulmonary tuberculosis). Written informed consents were obtained from all individuals. The study was approved by the Ethical Committee of Minia University, Faculty of Medicine.

Clinical and Functional Assessment

All patients underwent clinical examination, pulmonary function testing, and arterial blood gases evaluation. The smoking index was calculated. Lung volumes were obtained and calculated during the hospitalization.

Sputum Collection and Preparation

Paired sputum samples were taken from all patients, one sample was taken during exacerbation state and the other was taken at the stable state. Sputum was spontaneously expectorated into a sterile pot. Samples were transported directly to the Microbiology and Immunology Department, Faculty of Medicine, Minia University, where the microbiological and molecular analyses were performed. Sputum samples were processed by adding an equal weight of sputolysin (Sputasol, UK) and incubated for 30 minutes at 37°C during which they were vortexed for 5-10 seconds intermittently. A part of this suspension was stored at -80°C for molecular analysis and the rest was used for bacteriological examination.

Bacteriological Examination

Viable count: Ten-fold serial dilutions of the homogenized sample were made in brain heart infusion broth and 100 µl aliquots were plated out on the surface of a range of different media including blood agar, chocolate agar, Mac Conkey agar, and Cetrimide agar. These were incubated for 24 hours at 37°C. After incubation, bacterial colonies were counted and sub-cultured for identification by standard Methods [7]. The number of colonies forming units/ml sputum was calculated according to the number of colonies obtained and the dilution of the sputum.

Identification of the causative bacterial strains: The samples were examined microscopically after staining with Gram's stain and cultured directly on nutrient, blood, chocolate, Citrimide, MacConkey, and eosin methylene blue agar plates. The cultured plates were incubated aerobically at 37°C for 24-48 hours. Blood and chocolate agar plates were incubated at 35°C -36°C with 5% CO_2 for 48 hours for isolation of *S. pneumoniae* and *H. influenza* strains. Bacterial isolates were identified by colony morphology, Gram staining, and standard biochemical reactions according to Bergey's Manual of Systematic Bacteriology [8].

Antibiotic susceptibility testing: Susceptibilities of the isolated bacterial strains were determined to amoxicillin, amoxicillin-clavulanic acid, ceftriaxone, cefotaxime, ciprofloxacin, levofloxacin, erythromycin, vancomycin, amikacin, imipenem and linezolid (Oxoid, England). The test was performed using the disk diffusion method as recommended by the Clinical and Laboratory Standards Institute guidelines [9]. The results were interpreted as sensitive or resistant.

DNA Extraction and PCR Amplification

The homogenized sputum samples were centrifuged at 3,000 rpm for 15 minutes at room temperature. The supernatant was discarded, and the pellet was used for bacterial DNA extraction using Gene JET Genomic DNA Purification Kit (Thermo Scientific, INC) according to the manufacturer's instructions. The quantity of DNA was confirmed by Bio Photometer (Genova, UK). The integrity of the nucleic acids was determined visually by 1% agarose gels electrophoresis containing ethidium bromide and DNA was stored at -20°C (Table 1).

Table 1 16s rRNA PCR primers used in real-time PCR

Bacteria	Primers	Reference
S. pneumoniae	Forward (ACG CAA CTG ACGAGT GTG AC)	[10]
	Reverse (GAT CGC GAC ACCGAA CTA AT)	
K. pneumoniae	Forward (GAG GTC GGT GGTTCA AGT C)	[11]
	Reverse (TCG CAG TAA AGATGG TGG AG)	
P. aeruginosa	Forward (ATG GAA ATG CTGAAA TTC GGC)	[12]
	Reverse (CTT CTT CAG CTC GAC GCGACG)	
H. influenza	Forward (CTCAGATTGAACGCTGGCGGC)	[13]
	Reverse(TGACATCCTAAGAAGAGC)	

PCR amplification primers targeting the variable V3 region of 16S rRNA gene were applied, and the procedure was followed as per the manufacturer's protocol using Maxima SYBR Green PCR Master Mix (Thermo Scientific, US).

Each PCR reaction was performed in a 25 µl volume containing 100 ng of target DNA (3 µl), 2x SYBR Green master mix (12.5 µl), 0.3 µM each primer (1 µl), 50x reference dye low (0.5 µl) and PCR-grade water (up to 25 µl). An ABI 7500 instrument (Applied Biosystems, USA) was used for PCR amplification and fluorescence curve analysis.

Statistical Analysis

Statistical analysis was performed using the SPSS version 19.0 software for Windows (SPSS Inc., Chicago, Illinois, USA). Data were presented as mean and SD or number and percentage. A p-value less than 0.05 were considered statistically significant.

RESULTS

Patient Characteristics

Patient characteristics from 50 COPD patients were listed in Table 2. There were no statistically significant differences in clinical characteristics between COPD patients with positive sputum samples bacteria and patients with negative sputum samples bacteria by culture or PCR except for FEV/FVC ratio, which was significantly different between 2 groups (p=0.03).

Table 2 Clinical characteristics of COPD patients

Variables	All patients (N=50)	Patients with +ve bacteria (N=41)	Patients with -ve bacteria (N=9)	p-value
Sex	31 male (62%)	26 (63%)	5 (55.6%)	0.60
	19 female (38%)	15 (36.6%)	4 (44.4%)	
Age	59.48 ± 9.82	60.41 ± 8.12	55.22 ± 15.38	0.10
Smoking index	18.94 ± 16.53	19.90 ± 16.80	14.56 ± 15.38	0.30
PH	7.32 ± 0.05	7.32 ± .06	7.32 ± .03	0.80
$PaCO_2$	47.44 ± 11.92	48.66 ± 12.05	41.89 ± 10.14	0.10
PaO_2	62.34 ± 16.66	62.66 ± 16.77	60.89 ± 17.05	0.70
HCO_3	29.34 ± 6.85	29.39 ± 6.69	29.11 ± 7.97	0.90
SaO_2	73.86 ± 14.57	74.29 ± 15.11	271.89 ± 12.37	0.60
PF ratio	189.60 ± 38.15	193.00 ± 33.91	174.11 ± 53.26	0.10
FEV1	33.98 ± 10.89	33.59 ± 11.03	35.78 ± 10.68	0.50
FVC	50.42 ± 12.45	50.68 ± 12.53	49.22 ± 12.74	0.70
FEV/FVC	51.42 ± 6.19	50.56 ± 5.99	55.33 ± 5.89	0.03*

*Data was presented as percentage value or mean ± SD as appropriate

Prevalence and Load of Air Way Bacteria in COPD Patients

Paired sputum samples were taken from 50 COPD patients, one sample was taken during exacerbation state and the other was taken at the stable state. There were 41 samples out of 50 (82%) positive for bacteria at exacerbation compared with 12 samples out of 50 (24%) positive for bacteria (either by culture or PCR or both) at stable state samples (p=0.001). Also, the bacterial load was significantly higher (p=0.001) in the exacerbation state samples than stable state samples (mean ± SD =7 × 108 ± 2.1 and 4 × 106 ± 0.5 respectively).

Table 3 show types and percentages of bacteria detected in exacerbation and stable state samples by culture and PCR. The most commonly detected species was *H. influenza* followed *by S. pneumoniae, P. aeruoginosa,* and *K. pneumoniae*. In the exacerbation state samples, there were 3 samples which showed mixed bacterial growth by culture and 14 samples contained mixed bacteria by PCR. Also, there were 2 samples which showed mixed bacteria by PCR at stable state samples.

Table 3 Types and percentages of detected bacteria in exacerbation and stable state samples

Bacteria	Exacerbation state samples (N=50)		Stable state samples (N=50)		p-value
	Culture	PCR	Culture	PCR	
H. influenza	9 (18%)	11 (22%)	4 (8%)	5 (10%)	0.001*
S. pneumonia	4 (8%)	5 (10%)	1 (2%)	1 (2%)	
P. aeruginosa	7 (14%)	8 (16%)	2 (4%)	2 (4%)	
K. pneumoniae	6 (12%)	3 (6%)	2 (4%)	2 (4%)	
Mixed Bacteria	3 (6%)	14 (28%)	0 (0%)	2 (4%)	
Total	29 (58%)	41 (82%)	9 (18%)	12 (24%)	
*Samples with Mixed bacteria contained 2 types of bacterial species. All stable state samples that were reported positive for bacteria are also positive for bacteria in the corresponding exacerbation state samples					

Comparison between the Results of Culture and PCR

A comparison between the results of culture and PCR for the detection of different bacterial species in all samples either exacerbation or stable state samples were listed in Table 4. There were a total of 27 bacterial species detected by PCR and were not detected by culture and only 4 bacterial species were detected by culture and were not detected by PCR (p=0.001).

Table 4 Comparison between the results of culture and PCR in all samples

Bacteria	+ve Culture -ve PCR	-ve Culture +ve PCR	+ve Culture +ve PCR	p-value
H. influenza	0	7	16	0.001*
S. pneumoniae	0	13	6	
P. aeruginosa	1	5	9	
K. pneumoniae	3	2	6	
Total	4	27	37	
+v (Positive) and -ve (Negative)				

Antibiotic Resistance Pattern of Isolated Bacteria

Isolated strains show a high resistance pattern to the tested antibiotics as shown in Figure 1. Multi drug resistant strains (MDR) recorded in about 81%, 72%, 63% and 60% of *P. aeruoginosa, H. influenza, S. pneumoniae,* and *K. pneumoniae* strains respectively.

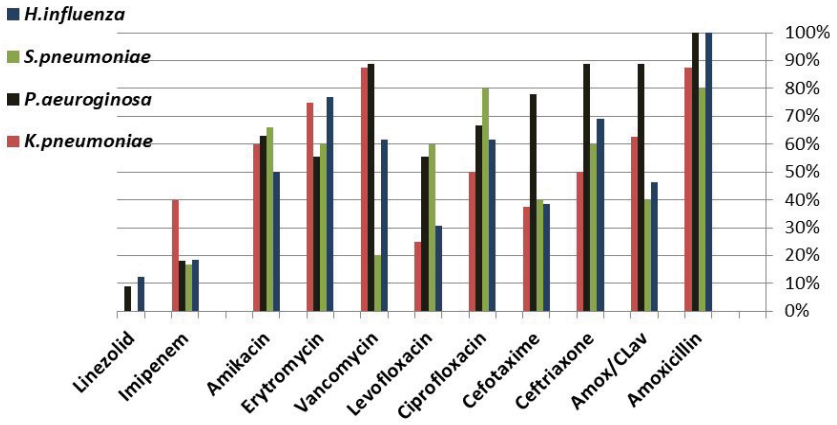

Figure 1 Antibiotic resistance pattern of the isolated bacterial strains. (High resistance pattern was recorded to Amoxicillin, Amoxicillin-clavulinic, Ceftriaxone, Cefotaxime, Ciprofloxacin, Levofloxacin, Vancomycin, Erythromycin, and Amikacin. Mild or no resistance was recorded to Imipenem and linzolid)

DISCUSSION

The current study included 50 COPD patients, 41 patients showed positive bacteria in sputum samples either by culture, PCR or both and 9 patients showed no bacteria in their sputum. The study deduced no statistically significant differences in baseline characteristics (including age, sex, smoking index, PH, PaO_2, $PaCO_2$, HCO_3, SaO_2, PF ratio, FEV1 or FVC) between these 2 groups of patients with only significantly lower FEV1/FVC ratio in the sputum bacterial positive group. Near to these results, Braeken, et al., found also no statistically significant differences regarding these baseline characteristics between sputum culture positive and sputum culture negative COPD patients [14]. From each patient, 2 sputum samples were withdrawn, one during exacerbation and the other during stable disease.

The current study elucidated that there were 29 samples out of 50 (58%) positive for bacteria at exacerbation compared with 9 samples out of 50 (18%) positive for bacteria (detected by culture) at stable state samples with a statistically significant difference between both groups (p=0.001). This agrees with Rosell, et al., as they found bronchial colonization in 29% of stable COPD patients and in 54% of patients with exacerbated COPD (p<0.001) [15]. This also agrees with Bari, et al., as their study revealed that out of 30 stable COPD patients, 6 (20%) showed positive sputum culture for bacteria, while out of 60 patients with acute exacerbation of COPD, 39 patients (65%) showed positive culture for bacteria [16]. Moreover, these results were in accordance with Garcha, et al., whose results elucidated that typical bacteria were more prevalent in exacerbation than stable-state paired samples: 30/52 (57.7%) vs. 14/52 (26.9%); p=0.001 [17].

Moreover, the current study elucidated that 41 samples out of 50 (82%) positive for bacteria at exacerbation compared with 12 samples out of 50 (24%) positive for bacteria at stable state samples (detected by PCR) with a statistically significant difference between both groups (p=0.001). Similar to these results, Garcha, et al., found that typical bacterial prevalence by PCR was higher in the exacerbation sputum samples compared with stable samples (74/131) bacterial isolates (56.5%) versus 107/242 (44.2%) respectively, (p=0.024) [17].

The current study showed that the most commonly detected bacterial species was *H. influenza* followed *by S. pneumoniae*, *P. aeruoginosa,* and *K. pneumoniae*. Similar to these results, a study of Rosell, et al., revealed a predominance of *H. influenza* and *P. aeruginosa*. Bari, et al., found that the organism commonly played a pathogenic role in acute exacerbations of COPD were *Pseudomonas* and *Klebsiella* [15,16].

Both *H. influenza* and *S. pneumonia* were detected more prevalently in exacerbation group than in stable state (9 (18%) and 11 (22%) vs 4 (8%) and 5 (10%) respectively for *H. influenza* and 4 (8%) and 5 (10%) vs 1 (2%) and 1 (2%) respectively for *S. pneumonia* "p=0.001"). These results agree with Garcha, et al., who found a significantly higher bacterial load of both *S. pneumoniae* and *H. influenza* at exacerbation than at stable state [17]. According to literature, the most common bacterial pathogens isolated in AECOPD are *H. influenzae, H. parainfulueza, S. pneumoniae* and *M. catharralis* [18].

Sharma, et al., concluded that out of the 78 cases of AECOPD where sputum culture revealed the growth of a bacterial organism, *S. pneumoniae* was the most common (13%) organism isolated [19]. *H. influenzae* and *M. catarrhalis* were isolated in 2% cases each. Collectively, Gram negative bacteria (GNB) were the main etiological agent responsible for AECOPD in 35.7% patients. Among GNB, *E. coli* (9.4%) was the most common isolated organism followed by Acinetobacter (8.1%), *P. aeruginosa* (7.5%) and *Klebsiella* (6.3%), while other studies have implicated *P. aeruginosa* and *Klebsiella* species as the most common organisms responsible for AECOPD [20,21].

Sputum culture and PCR were compared for the detection of bacterial species in sputum samples. PCR detected more bacterial species than sputum culture as a total of 27 bacterial species were detected by PCR, and were not detected by culture and only 4 bacterial species were detected by culture, and were not detected by PCR, while 37 bacterial species were detected by both culture and PCR (p=0.001) and it was more detectable for individual species "*H. influenza, S. pneumonia, P. aeruginosa,* and *K. pneumonia*". This is in accordance with Garcha, et al., as their study revealed that PCR was far more discriminatory at detecting typical bacteria than culture (105/177) positive samples (59.3%), vs 43/177 (24.3%), p<0.001) and it was also more discriminatory for each bacterial species (*H. influenza, S. pneumonia,* and *M. catarrhalis*) [17].

It is known that *H. influenzae* is able to persist intra-cellularly in the respiratory tract, with possible resultant culture-negative sputum [22]. A small number of samples (4 samples) was detected by culture not detected by PCR. This can

be explained by a number of potential explanations, including technical issues with the primer targets, operator error or sampling [17].

The advantage of PCR is that it gives a higher rate of detection than culture [17]. Using this technique, the current study demonstrates that exacerbations in COPD are characteristically associated with both increased prevalence and load of typical bacteria. This confirms previous data suggesting that the increase in load and acquisition of bacteria seen at exacerbation is driving such exacerbations [23]. Microbiological culture is, however, useful in other aspects of bacteriology, such as in the identification of antibiotic resistance. One limitation of PCR microbiological technique is that they can detect both viable and non-viable bacteria, while culture detects viable bacteria alone [17].

Regarding drug sensitivity, the current study revealed that isolated strains showed high resistance pattern to the tested antibiotics and that Multi drug resistant strains (MDR) were recorded in about 81%, 72%, 63% and 60% of *P. aeuroginosa*, *H. influenza*, *S. pneumoniae,* and *K. pneumoniae* strains respectively. This coincides with the recent report by Hassan, et al., who reported that most of the *H. influenza* (77%), *S. pneumoniae*, (73%) and *K. pneumonia* strains (62%) were MDR [24]. These findings are against the results recorded with Sharma, et al., as they found that antibiogram of isolated organisms revealed that usual organisms considered responsible for AECOPD, like *S. pneumoniae*, *H. Influenzae,* and *M. catarrhalis*, were sensitive to commonly used antibiotics: fluoroquinolones, cephalosporin, aminoglycoside and piperacillin-tazobactam [19]. This may be due to different communities with maluse of antibiotics in our locality.

CONCLUSION

This study has shown that airway bacterial prevalence and load increase at COPD exacerbations, and there is a significant relationship between lower airway bacterial colonization and COPD.

DECLARATIONS

Conflict of Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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