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Incidence of Biofilm Formation in ET Tube and Correlation with Occurrence of VAP in a Tertiary Care ICU

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Abstract **Introduction:** VAP is one of the most frequent hospital-acquired infections occurring in mechanically ventilated patients and is associated with increased mortality, morbidity, and health-related costs. Bacterial biofilm has been observed universally on the surface of endotracheal tubes in mechanically ventilated patients. Some data show a good concordance between bacterial colonization of the airway and microbial findings in the biofilm. Even the same microorganisms causing VAP could be found in the ETT biofilm leading to the potential implication of biofilm in the genesis of VAP. **Aims and Objectives:** The objective was to investigate the involvement of ETT biofilm in VAP pathogenesis. **Materials and Methods:** This observational descriptive study was conducted in Department of Pulmonary Medicine, D.Y Patil Hospital, Nerul, Navi Mumbai. Approval of Institutional Ethics Committee was taken before start of the study. A written signed informed consent was taken prior to enrolling the subjects in the study. Patients requiring mechanical ventilation for at least 24 hours were included in the study. The following data were recorded in a standardized form: age and sex of the patient, cause of mechanical ventilation, duration of ventilation, manoeuvres to prevent VAP, the occurrence or not of nosocomial pneumonia, together with data of pathogen isolated in respiratory samples and/or ETT biofilm, antibiotic therapy and sensitivity patterns. **Statistical Method:** Descriptive and analytical statistics was done. The data was analyzed using statistical software (IBM SPSS V20.1, IBM Corporation, Armonk, NY, USA). The results are expressed as mean ± standard deviation and proportions. Categorical variables were compared using chi-square tests. The statistical significance was determined at $p<0.05$.**Results:** A total of 55 subjects fulfilling the inclusion criteria were participated in the study. The mean age of the study population was 46.56 ± 19.79 with a range of 18 to 87 years. There were 23 (41.8%) males and 32 (58.2%) female's subjects in the study population. The patients were intubated for mainly three reasons – AHF, ARF and CVA. The diagnosis of the ventilated patients was as follows – pneumonia (25.4%), ARDS (18.2%), cerebral stroke (16.4%), septic shock (16.4%), hemorrhagic shock (9.1%), cardiac arrest (7.3%), cardiopulmonary failure (5.4%) and interstitial lung disease (1.8%). The mean duration of intubation was 10.23 ± 1.55, ICU days was 13.45± 1.34 and hospital days was 19.72 ± 2.84. Out of the 55 ventilated patients, 9 (16.3%) patients developed VAP. Biofilm was found positive in 30 (54.5%) patients. In the present study the three most common organisms found in ET tube were Acinetobacter (23.6%), klebsiella (18.2%), E.Coli (7.3%). The other organisms found were Pseudomonas (7.3%), Candida Albicans (3.6%) and staphylococcus (1.8%). The incidence of VAP development in biofilm positive cases was 30%. **Conclusion:** Biofilms are highly organized microbial communities which in vivo play an important part in evading the defence mechanism and obstinate the antimicrobial therapy. The precise link between biofilm productions in mechanically ventilated patients is still obscure apart from some limited studies which have described role of a specific pathogen or virulence factor. The present study, thus, showed that majority of the pathogens isolated possessed capability to produce biofilm.

Keywords: *VAP, EET, biofilm, nosocomial pneumonia, hospital acquired pneumonia*

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1. Introduction

Ventilator-associated pneumonia (VAP) is a type of nosocomial pneumonia that occurs in patients who receive mechanical ventilation and is usually acquired in the hospital setting approximately 48-72 hours after mechanical ventilation, characterized by the presence of a new or progressive infiltrate, signs of systemic infection (fever, altered white blood cell count), changes in sputum characteristics, and microbiologically by detection of a

causative agent. [1,2,3] VAP is one of the most frequent hospital-acquired infections occurring in mechanically ventilated patients and is associated with increased mortality, morbidity, and health-related costs. Several risk factors have been reported to be associated with VAP, including the duration of mechanical ventilation, and the presence of chronic pulmonary disease, sepsis, acute respiratory distress syndrome (ARDS), neurological disease, trauma, prior use of antibiotics, and red cell transfusions. The complex interplay between the endotracheal tube, presence of risk factors, virulence of the invading bacteria and host immunity largely determine the development of VAP. [4,5]

Infection in VAP develops by direct entry of bacteria to lower respiratory tract, which may be innate flora of oropharynx or those present in the hospital *via*: (a) micro aspiration, which can occur during intubation itself; (b) development of a biofilm overloaded with bacteria (typically Gram-negative bacteria and fungal species) within the endotracheal tube; (c) pooling and trickling of secretions around the cuff; and (d) impairment of mucociliary clearance (disrupting the cough reflex, thus promoting the accumulation of tracheo-bronchial secretions and increasing the risk of pneumonia). [1,6,7] In addition, the insertion of an ETT could produce injury and inoculate endogenous oropharyngeal bacteria in the low airway tract. [8]

The type of organism that causes VAP usually depends on the duration of mechanical ventilation. In general, early VAP is caused by pathogens that are sensitive to antibiotics, whereas late onset VAP is caused by multi-drug resistant and more difficult to treat bacteria. Geographical variation is being detected for the exact prevalence of MDR organisms and even within institutions at one place. [1,3,6] Formation of biofilm on the surface of ETT is an almost universal phenomenon and it has been related to the pathogenesis of ventilator-associated pneumonia (VAP). Due to the role of ETTs in the pathophysiological development of VAP, some authors suggest that it should be renamed ETT-associated pneumonia. [9,10]

Microorganisms attach to synthetic surfaces, multiply and develop biofilms characterized by the generation of an extracellular polymeric substance or matrix that has been well documented with scanning electron microscopy (SEM) studies. [11,12] Biofilms have great importance for public health because of their role in certain infectious diseases and their role in a variety of device-related infections. [13,14,15] In those device-related infections, biofilms have been also involved in bacterial antibiotic resistance that depends on multi-cellular strategies. [12,13] This resistance implies, in most of the cases, the necessity of device withdrawal in order to achieve clinical and microbiological cure.

Bacterial biofilm has been observed universally on the surface of endotracheal tubes in mechanically ventilated patients. Some data show a good concordance between bacterial colonization of the airway and microbial findings in the biofilm. Even the same microorganisms causing VAP could be found in the ETT biofilm leading to the potential implication of biofilm in the genesis of VAP. [16,17,18] In fact, a study has demonstrated the efficacy of a novel silver-coated ETT in decreasing the incidence of microbiologically confirmed VAP, although no statistically significant differences between-group were observed in duration of intubation, intensive care unit stay, and hospital stay or mortality.

This current investigation aimed to study the presence of biofilm formation by various organisms such as Escherichia coli, Klebsiella pneumoniae, Acinetobacter baumannii and Pseudomonas aeruginosa isolated from mechanically ventilated and VAP developed patients. The objective was to investigate the involvement of ETT biofilm in VAP pathogenesis.

2. Materials and Methods

This prospective observational study was conducted in Department of Pulmonary Medicine, D.Y Patil Hospital, Nerul, Navi Mumbai. Approval of Institutional Ethics Committee was taken before start of the study. A written signed informed consent was taken prior to enrolling the subjects in the study. A total of 55 consecutively admitted patients to Medical Intensive Care Unit that required mechanical ventilation for at least 24 hours were included in the study. Reventilated patients and patients who refuse to be part of the study were excluded.

2.1. Study Procedure

The following data were recorded in a standardized form: age and sex of the patient, cause of mechanical ventilation, duration of ventilation, manoeuvres to prevent VAP, the occurrence or not of nosocomial pneumonia, together with data of pathogen isolated in respiratory samples and/or ETT biofilm, antibiotic therapy and sensitivity patterns.

Manoeuvres to prevent VAP, such as elevation of the head of the bed and subglottic aspiration, were performed in all our patients. Selective digestive decontamination protocol consisted of intravenous cefotaxime 1 g/8 h for three days plus nasogastric administration of 0.5 g of 2% antibiotic gel with 100 mg polymyxin E+ 80 mg tobramycin + 500 mg amphotericin B every 6 h while duration of mechanical ventilation.

The ET tubes were sent to the microbiology lab for processing and identification of growth and biofilm production by organism. The ET tubes were inoculated with Thioglycollate broth along with blood agar, Mac Conkey agar and chocolate agar.

The plates were incubated at 37°C for 24 hour and growth of organism was identified using biochemicals. The organisms were then inoculated on Congo red agar to check for presence of biofilm.

The Congo red agar method is a qualitative assay for detection of biolfim producing organisms by colour change of colonies inoculated on CRA medium. The CRA medium is constructed by mixing 0.8g of Congo red and 36g of sucrose to 37g/L of Brain heart infusion (BHI) agar. After incubation for 24 h at 37C, morphology of colonies that have undergone to different colours is differentiated as biofilm producers or not. Black colonies with a dry crystalline consistency indicate biofilm producers, whereas colonies retained pink are non-biofilm producers.

2.2. Respiratory Samples

We performed surveillance sampling of endotracheal aspirates (ETA) on 2nd and 8th day after intubation with analysis in the Microbiology which was quantitatively cultured

2.3. Definitions

VAP was defined as previously reported. [19] The diagnosis of VAP in our study was: a) clinical: new or progressive lobar infiltrates > 48 hours after intubation, and two or more of the following minor criteria (fever, leukocytosis/leucopoenia, and purulent respiratory secretions) and was microbiologically confirmed. [20] Microbiology of VAP was determined by mini-bronchial-alveolar lavage (miniBAL) obtained after clinical suspicion and confirmed VAP when yielded > 1,000 UFC/ml. Empirical therapy and the management of patients were based in ATS/IDSA guidelines. [21] We considered as early-onset VAP those episodes that were initiated four days or less upon intubation. [21,22,23,24] Appropriate antimicrobial therapy was defined as coverage of all pathogens isolated by the antimicrobial therapy administered at the onset of VAP determined by the sensitivity pattern in the anti-biogram. [25]

Microbial persistence was defined as the persistence of the causative microorganism of the VAP episode in at least two successive respiratory samples, despite 72 hours of proper antibiotic therapy irrespective of colony counts. [26]

VAP relapse was defined as reported: [27] (a) occurrence at least 72 h after clinical resolution; (b) positive bronchoscopic quantitative culture for previously isolated strain; (c) evidence for a new infiltrate on the chest X-ray; (d) two of the following: fever > 38°C; white blood cell (WBC) count > 10,000/mm^3; or purulent respiratory secretions; and (e) absence of evidence of a new extra pulmonary source of infection. The definition of treatment failure included at least one of the following 72 hours after the initiation of treatment: (a) failure to improve the PaO2/FiO2 ratio or need of intubation because of pneumonia; (b) persistence of fever (> 38°C) or hypothermia (< 35°C) and purulent respiratory secretions; (c) worsening of pulmonary infiltrates (> 50%); (d) occurrence of septic shock or multiple organ dysfunction not present at the onset of pneumonia. [27,28,29]

Clinical resolution was defined as stated elsewhere: [30] including: (1) roentgen graphic improvement, (2) normothermia, (3) WBC ≥3,000/mm^3 or WBC ≤12,000/mm^3, (4) completion of a course of antibiotic therapy.

2.4. Statistical Method

The descriptive and analytical statistics were done. All the data was analyzed using statistical software (IBM SPSS V20.1, IBM Corporation, Armonk, NY, USA). Results were expressed as mean ± standard deviation and proportions. Comparisons between categorical variables were performed with Fisher's exact and chi-square tests. The statistical significance was determined at $p<0.05$.

3. Results

A total of 55 patients fullfilling the inclusion criteria were enrolled in the study. The mean age of the study population was 46.56 ± 19.79 with a range of 18 to 87 years. There were 23 (41.8%) males and 32 (58.2%) females subjects in the study population. The patients were intubated for mainly three reasons – AHF, ARF and CVA. The diagnosis of the ventilated patients was as follows – pneumonia (25.4%), ARDS (18.2%), cerebral stroke (16.4%), septic shock (16.4%), hemorrhagic shock (9.1%), cardiac arrest (7.3%), cardiopulmonary failure (5.4%) and interstitial lung disease (1.8%).

The mean duration of intubation was 10.23 ± 1.55, ICU days was 13.45± 1.34 and hospital days was 19.72 ± 2.84. ETA culture report at 2nd and 8th day of intubation is presented in Table 1 & Table 2. The drug sensitivity report against the identified micro-organisms is given in Table 4. Nine patients developed VAP out of the total 55 intubated patients (16.3%). The incidence of biofilm formation was found to be 54.5% in ET tube. In the present study the three most common organisms found in ET tube were Acinetobacter (23.6%), klebsiella (18.2%), E.Coli (7.3%). The other organisms found were Pseudomonas (7.3%), Candida Albicans (3.6%) and staphylococcus (1.8%) Table 3. In this study the occurrence of VAP in biofilm positive cases was significantly correlated (p=0.003) Table 5. Out of the total 21 biofilm positive cases 9 (30.0%) had VAP. Nine patients could not be saved, 2 patients took DAMA and 44 patients were discharged at the end of the study.

Table 1. ETA culture at 2nd day

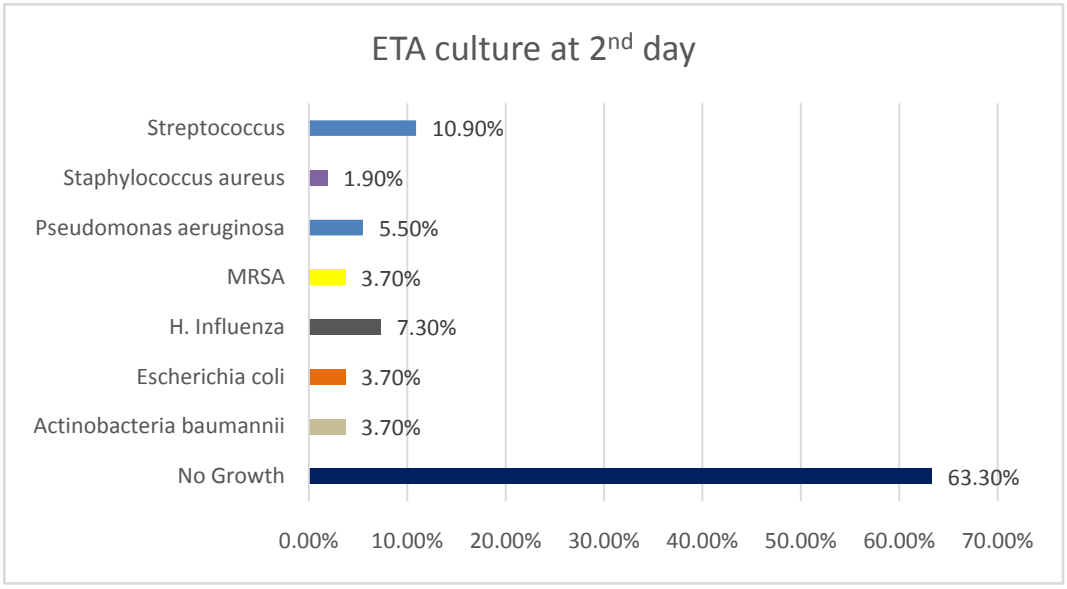
ETA Culture 2nd Day	N	%
No Growth	35	63.3
Acinetobacter baumannii	2	3.7
Escherichia coli	2	3.7
H. Influenza	4	7.3
MRSA	2	3.7
Pseudomonas aeruginosa	3	5.5
Staphylococcus aureus	1	1.9
Streptococcus	6	10.9

Table 2. ETA culture at 8th day

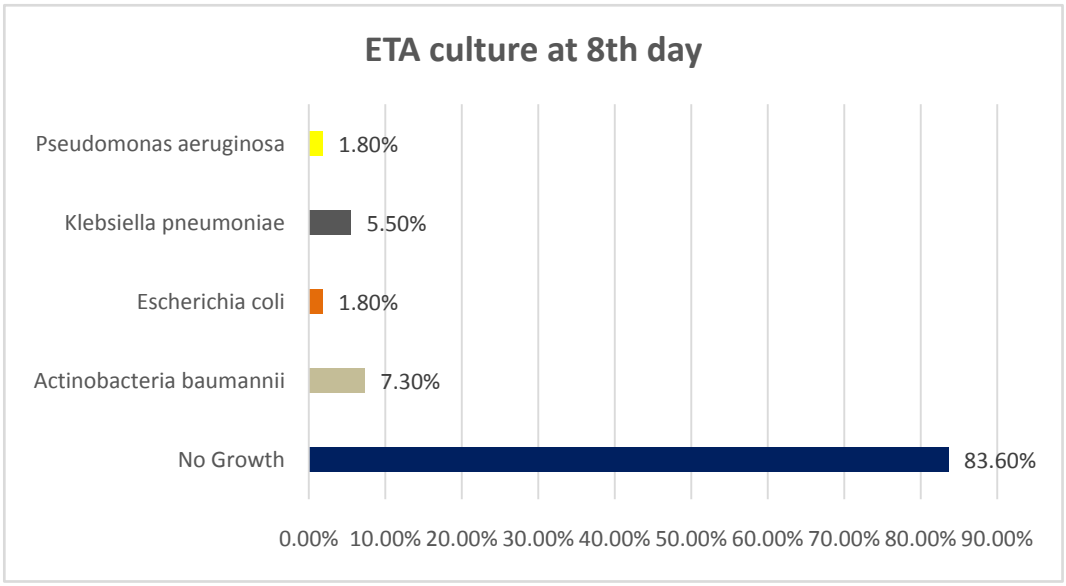
ETA Culture 8thDay	N	%
No Growth	46	83.6
Acinetobacter baumannii	4	7.3
Escherichia coli	1	1.8
Klebsiellapneumoniae	3	5.5
Pseudomonas aeruginosa	1	1.8

Table 3. Distribution of patients according to microbial growth present in ET tube

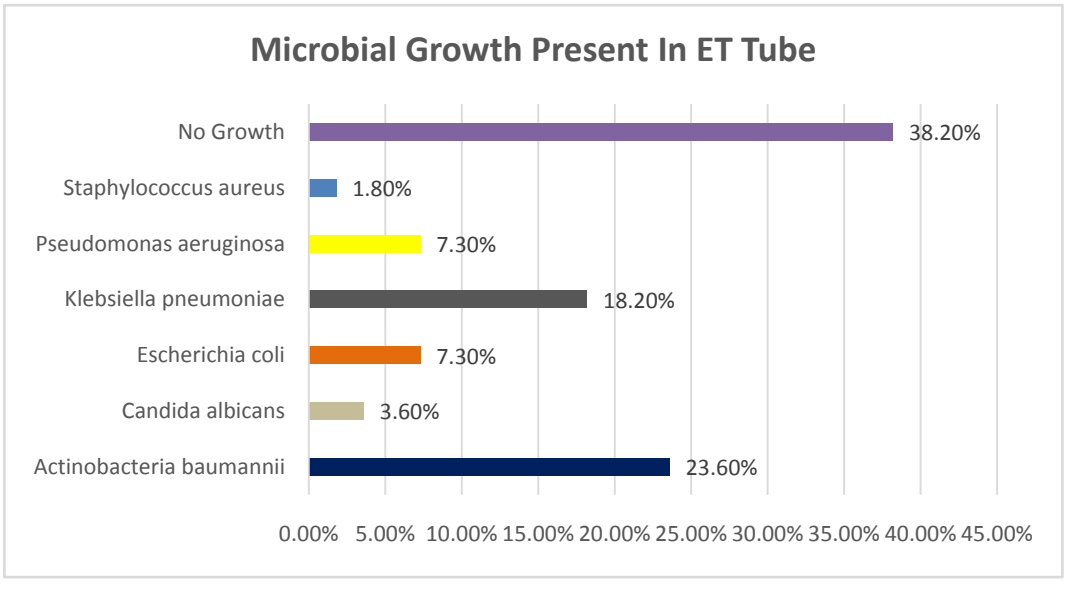
Variables	N	%
Acinetobacter baumannii	13	23.6
Candida albicans	2	3.6
Escherichia coli	4	7.3
Klebsiellapneumoniae	10	18.2
Pseudomonas aeruginosa	4	7.3
Staphylococcus aureus	1	1.8
No Growth	21	38.2
Total	**55**	**100.0**



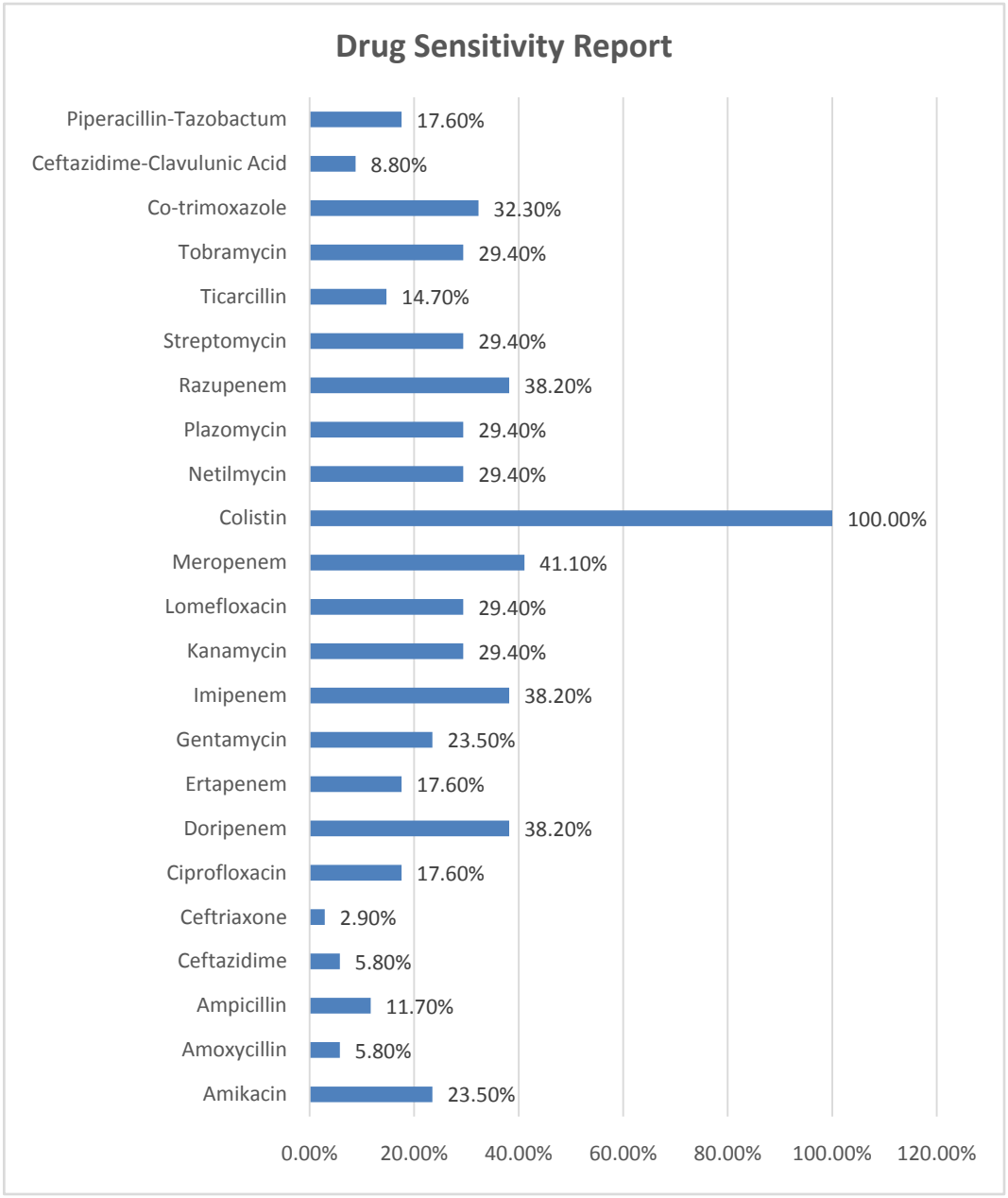
Bar Diagram 1. ETA culture at 2nd day



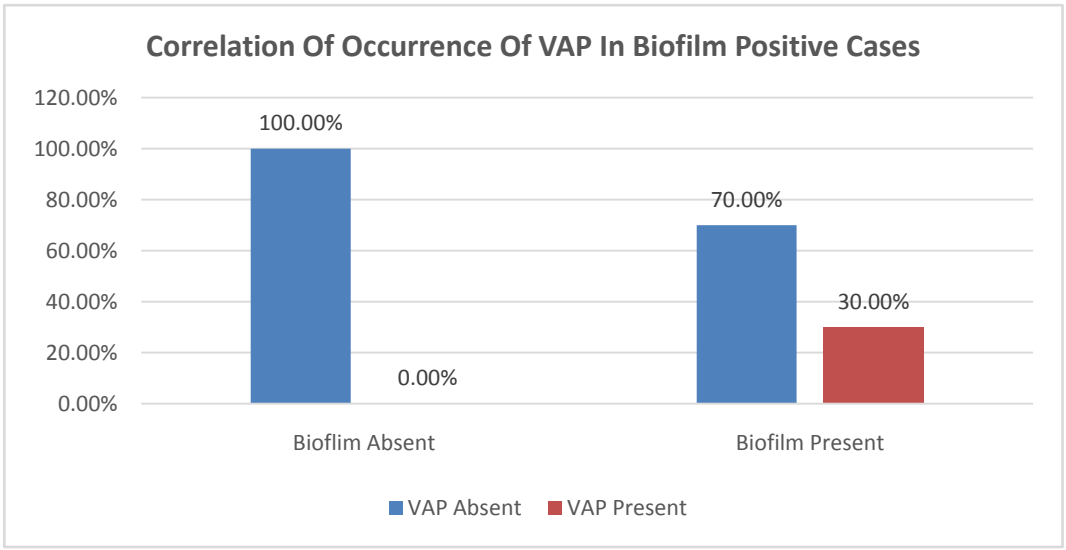
Bar Diagram 2. ETA culture at 8th day



Bar Diagram 3. Distribution of patients according to microbial growth present in ET tube



Bar Diagram 4. Drug sensitivity report against the identified micro-organisms



Bar Diagram 5. Correlation of occurrence of VAP in biofilm positive cases

Table 4. Drug sensitivity report against the identified micro-organisms

Drug Sensitivity	N	%
Amikacin	8	23.5
Amoxycillin	2	5.8
Ampicillin	4	11.7
Ceftazidime	2	5.8
Ceftriaxone	1	2.9
Ciprofloxacin	6	17.6
Doripenem	13	38.2
Ertapenem	6	17.6
Gentamycin	8	23.5
Imipenem	13	38.2
Kanamycin	10	29.4
Lomefloxacin	10	29.4
Meropenem	14	41.1
Colistin	34	100.0
Netilmycin	10	29.4
Plazomycin	10	29.4
Razupenem	13	38.2
Streptomycin	10	29.4
Ticarcillin	5	14.7
Tobramycin	10	29.4
Co-trimoxazole	11	32.3
Ceftazidime-Clavulunic Acid	3	8.8
Piperacillin-Tazobactum	6	17.6

Table 5. Correlation of occurrence of VAP in biofilm positive cases

Biofilm	VAP		χ^2-value	P-value[#]
	Absent	Present		
Present	21 (70.0)	9 (30.0)	8.967	0.003
Absent	25 (100.0)	0 (0.0)		
Total	46 (83.6)	9 (16.4)		

[#]P-value derived from chi-square test.

4. Discussion

Ventilator associated pneumoniae is a nosocomial infectious affliction which presents after 48 hours to 72 hours of stay in the hospital. In the present investigation endotracheal aspirate from 55 ICU admitted and mechanically ventilated patients was cultured for any gram negative organism and their capacity to form biofilm was assessed.

In the present study the three most common organisms found in ET tube were Acinetobacter (23.6%), klebsiella (18.2%), E.Coli (7.3%). The other organisms found were Pseudomonas (7.3%), Candida Albicans (3.6%) and staphylococcus (1.8%). Our report is analogous to other published studies from United States and other countries [30,31,32] whereby most frequent bacterial agent associated with VAP has been gram negative organisms. However, frequency of bacterial isolates differs geographically and even in local regions. All the above research studies performed revealed either *K. pneumoniae, P. aeruginosa* or *A. baumannii* as the most frequent bacterial isolates.

The present study evaluated the antibiotic susceptibility of all bacterial isolates by using the standard guidelines. Most of the gram negative organisms were resistant to cephalosporins, quinolones and carbapenems. Our study is compatible to a research performed in Pakistan which found 95.6% isolates resistant towards imipenem. A study done in Iran also showed similar results.

Resistance pattern of *A. Baumannii* towards several antibiotics was found similar to another study performed by Ibrahim and colleagues [33] and also to a study done by Shahrokhi E et al. The only antibiotic to which this organism did not develop resistance was colistin. *K. pneumoniae* was found as the second most common bacterial agent of VAP in our study. This organism was found mostly resistant to imipenem, ceftriaxone, ceftazidime, cefotaxime and ciprofloxacin. Almost similar finding has been reported from Bangladesh [34] and other research study [35] which found this organism to be resistant to not only the above mentioned antibiotics but also to gentamicin and amikacin. *P. aeruginosa* showed high level resistant to imipenem, ofloxacin and low level resistance towards piperacillin-tazobactum in our investigation. Other published studies conducted in year 2010 [36] and 2014 [37] furnishes high level resistance of this organism to most of the antibiotics, specially 45% of their isolates were resistant to piperacillin-tazobactum in comparison to our results. [37] *E. Coli* is another gram negative organism which has developed resistance to many antibiotics and this is a matter of concern as resistance genes are easily transferable to other strains. In our study, *E. coli* isolates demonstrated moderate resistant to extended spectrum cephalosporin and aminoglycoside. Our results are compatible to another published study [38] but the results of others study showed widespread resistance of the isolates to all the antibiotics, except nitrofurantoin.

Biofilms are common concern in medicine as they develop commonly on medical devices and they can also form on living tissues, as in the case of endocarditis. Biofilms grow slowly, in one or more locations, and biofilm infections are often slow to produce overt symptoms. [39] The first views of ETT biofilm were described in 1986, using electronic microscopy, and an integrally covered inner side of the tube was found in 84% of cases. [40] Berra and co-workers [41] studied ETT biofilm from animals ventilated for 24 h, and found 750 μm of thickness using electron microscopy and 65 μm with confocal microscopy. In a clinical trial using confocal microscopy, the same authors demonstrated an accumulation of biological material within the ETT, ranging from 0 to 700 μm. [42] In this study the occurrence of VAP in biofilm positive cases was significantly correlated (p=0.003). Out of the total 21 biofilm positive cases 9 (30.0%) had VAP. In accordance with some other studies performed on this topic, we found a high concordance between bacteria colonizing the airway and subsequently causing VAP. [43-47] In a study conducted by Gil Perotin et al, [48] only 14 patients developed pneumonia in their study group despite the high prevalence of airway colonization and biofilm on ETT,. They suggested that biofilm formation and airway colonization were necessary but not sufficient for VAP development. Among the known VAP risk factors, they found that days of mechanical ventilation and airway colonization by A. baumannii and P. aeruginosa increased the risk for late onset VAP. [49,50]

The pathogenesis of VAP is multifactorial. Aspiration of oropharyngeally-contaminated secretions or leakage of

bacteria around the endotracheal tube cuff is probably the primary route of bacterial colonization of the distal airways, but biofilm could also contribute, [51-55] and several studies have attempted to prevent, eliminate, or decontaminate the biofilm. [56]In vitro studies demonstrated that tubes covered with an antiseptic solution did not present biofilm [57] or did so to a lesser degree. These results were confirmed experimentally with tubes covered with silver sulfadiazine and chlorhexidine, [42] or silver-coated ETT. [58] Berra et al [43] have concluded that silver-coated ETT in ICU patients decreased overall bacterial colonization. The North American Silver-Coated Endotracheal Tube (NASCENT) study included 2,003 subjects intubated with either a standard tube or a silver-coated ETT, [33] showing a decrease in the VAP rate from 7.5% to 4.8% without effect on mechanical ventilation duration or mortality. A removable stalk with an inflatable balloon to eliminate adhered mucus inside the ETT has also been proposed. [41,43] In a short clinical trial, this device appeared safe, and could prevent or reduce secretion deposits. [59]

5. Conclusion

Biofilms are highly organized microbial communities which in vivo play an important part in evading the defence mechanism and obstinate the antimicrobial therapy. The precise link between biofilm productions in mechanically ventilated patients is still obscure apart from some limited studies which have described role of a specific pathogen or virulence factor. The present study, thus, showed that majority of the pathogens isolated empirical therapeutic agents and possessed capability to produce biofilm.

In this study the occurrence of VAP in biofilm positive cases was significantly correlated. Out of the total 21 biofilm positive cases 9 (30.0%) had VAP. These observations suggest that biofilm formation could contribute lower respiratory infection and help the organisms evade antimicrobial pressure and emergence of multidrug or extensive drug resistant microorganism. In the present study the three most common organisms found in ET tube were Acinetobacter (23.6%), klebsiella (18.2%), E.Coli (7.3%). The other organisms found were Pseudomonas (7.3%), Candida Albicans (3.6%) and staphylococcus (1.8%). The condition may lead to development of nosocomial infection too.

In summary, we provided data regarding the presence of biofilm in the high to moderate antibiotic resistant gram negative bacteria causing VAP which reveals predilection towards biofilm and is a concern in medical practice. It also necessitates the development of new strategies to impair their ability to persist in biofilm environment.

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