



Biofilm production and multidrug resistant bacterial isolates in ventilator associated pneumonia

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Abstract

Context: Ventilator-associated pneumonia is the second most common complication among all types of nosocomial infections. Mechanical ventilation predisposes to formation of a biofilm which worsens the prognosis because of increased multidrug resistant isolates implicated in formation of biofilm.

Aim of the Study: The study was conducted to find out the relationship between duration of mechanical ventilation, biofilm formation, and antibiotic resistance among VAP pathogens.

Study Design and Methods: A descriptive analytical study of 150 clinically suspected VAP patients was done. Patients were divided into Group I and II based on intubation duration for 1–5 days and more than 6 days, respectively. Endotracheal aspirate was collected from clinically diagnosed cases and processed as per standard microbiological techniques. Bacterial counts $\geq 10^6$ CFU/mL for quantitative cultures were considered significant. Biofilm production was detected by tissue culture plate method. Multivariate analysis was done to find out the association of the various factors.

Results: Klebsiella pneumoniae was the predominant bacteria isolated followed by Acinetobacter baumannii. Among Gram negative bacteria 66.8% were β -lactamase producers. In biofilm production by tissue culture method, Group I patients, 72.4% of the isolates showed either strong / moderate biofilm formation and in Group II patients, 92.3% of the isolates showed either strong / moderate biofilm formation. Multivariate analysis revealed that bacteria isolated from VAP occurring after 5 days of mechanical ventilation among prior antibiotic-treated patients were resistant to all the antibiotics tested.

Conclusion: Bacterial aetiology, prolonged intubation, biofilm formation, and drug resistance have ramification on outcome of VAP. Hence removal of ET tube in regular intervals should be considered with a proper choice of antimicrobial treatment or using ET tube coated with drugs/ biomaterials that discourage biofilm formation may be explored.

Keywords: Ventilator-associated pneumonia, Biofilm, Multidrug-resistant bacteria.

Introduction

Nosocomial infections are a burden to both healthcare institutions and patients. In developed countries, nosocomial infections account for up to 7% and in developing countries, the incidence of nosocomial infections is 10%. [1,2] They lead to prolonged stay, mortality, morbidity and significant economic burden. [1] While hospitals face shortage of beds, and economic loss indirectly, patients face direct consequences. Ventilator-associated pneumonia (VAP)

is the second commonest among the HAIs. [3,4] VAP is defined as pneumonia that develops after 48-72 hours of endotracheal intubation, 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 detection of a pathogen. [5] Endotracheal intubation is the most important risk factor, followed by prolonged stay and underlying diseases. The presence of an endotracheal tube (ETT) in ventilated

bacteria in the lower airway. Finally, formation of biofilm on the surface of ETT is an almost universal phenomenon, and it boosts the pathogenesis of VAP. Microorganisms attach to synthetic surfaces, multiply and develop biofilms characterized by the generation of an extracellular polymeric substance or matrix that helps bacteria to linger in a favorable micro environment rather than being swept away by the current. [7] The biofilm associated infections pose a greater challenge in treatment because of associated multidrug resistant bacteria (MDR). [8] Since biofilms are associated with development of VAP, ETT withdrawal in case of VAP patients has led to clinical improvement of cases. This is because of the removal of the foreign substance which hinders the clearance of the lower airway and decreased colonization. Some data show a good concordance between bacterial

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patients impairs mucociliary clearance and disrupts the cough reflex, thus promoting the accumulation of tracheobronchial secretions and increasing the risk of pneumonia. [6] In addition, the insertion of an ETT could produce injury and inoculate endogenous oropharyngeal

Table 1: Bacterial profile of ventilator associated pneumonia In Group-I and Group-II

Bacteria isolated	Group-I	Group-II
<i>Klebsiella pneumoniae</i>	40	9
<i>Acinetobacterbaumannii</i>	19	23
<i>Citrobacterfreundii</i>	14	12
<i>Staphylococcus aureus</i>	20	-
<i>Pseudomonas aeruginosa</i>	13	4
<i>E. coli</i>	10	2
<i>Streptococcus pneumoniae</i>	5	2
<i>Serratiaspp</i>	1	-

colonization of the airway and microbial findings in the biofilm. The bacteria in a biofilm are protected from killing by antibiotics. [4] The mechanisms by which bacteria protect themselves in a biofilm differ from that of the general mechanism. These methods include decreased antibiotic penetration, nutrient limitation and slow growth among others. [9] However, no attempt has been performed to assess the relationship among biofilm, microbial persistence, and outcome of the VAP episode. The present study was undertaken to determine the relationship between antibiotic resistance of ETT biofilm and pulmonary pathogens in VAP.

Subjects and Methods

The study was approved by the Institutional Ethics Review Board of S. S. Institute of Medical Sciences and Research Centre. Informed consent was taken before sample collection. Clinically suspected patients according to CDC criteria scored by the Chronic Pulmonary Infection Score (CPIS) were included in the study. [3] Patients with pneumonia before mechanical ventilation or within 48 hours of mechanical ventilation, patients with adult respiratory distress syndrome, cavitory lung disease based on chest X-ray findings, primary lung cancer, or another malignancy metastatic to the lungs and cystic fibrosis, tuberculosis patients and patients with acquired, induced or congenital immunodeficiency, leucopenia < 1000 cells/mm³, and neutropenia < 500 cells/mm³ were excluded from the study.

Groups

Patients were divided into Group I and II based on intubation duration. Group I intubated for 1–5 days and Group II intubated for more than 6 days.

Specimen collection

Endotracheal aspirate (ETA) was collected from clinically diagnosed cases. ETA was collected using two Catheters where in a Ramson's 8F suction catheter was guided through a Ramson's 14F suction catheter and gently introduced through the ETT for approximately 24cm. [10] The sample was gently aspirated without instilling saline, and the suction catheters were withdrawn. The sample was transferred into a clean labeled container. The sample was immediately transported to the laboratory for microbiological analysis. ETA was homogenized by vortexing for 1 min followed by centrifugation at 3000 rpm for 10 min. 1 mL of sample was diluted in 9 mL of 0.9% sterile saline (1 in 10).

The specimen was plated on sheep blood agar and MacConkey agar using nichrom wire loop with internal diameter of 4mm, which holds 0.01 mL of homogenized ETA secretions. Both plates were incubated at 37°C for 16–18h. Threshold of bacterial counts ≥ 10⁶ CFU/mL for quantitative cultures from ETA secretions was considered for diagnosis of VAP. Bacteria were identified by standard microbial techniques [11]. The antimicrobial susceptibility testing was performed by Kirby–Bauer disc diffusion method [12] according to the criteria put forward by the Clinical Laboratory Standards Institute. [13] Suspected extended-spectrum betalactamases (ESBLs) producing organisms were confirmed by double disk synergy test. [14] Detection of plasmid-mediated AmpC was done by the AmpC disk test, and the isolates showing reduced susceptibility to carbapenems (imipenem and meropenem) were selected for detection of metallo-beta-lactamases (MBLs) enzymes by imipenem-EDTA disk method [14]. MRSA was detected using cefoxitin discs by disc diffusion method. For

production was detected by tissue culture plate method; Overnight culture of the isolate from nutrient agar plate was inoculated into Trypticase soy broth (TSB). The primary inoculum was inoculated in TSB with 1% glucose prepared in different dilutions (1:20, 1:40, 1:60, 1:80, and 1:100) and loaded into 96 wells flat bottom microtitre plate. Plates were covered and incubated at 37°C for 24 hours in aerobic condition. The wells were then decanted and washed three times with Phosphate buffer saline (PBS). After washing, fixation was done by adding methanol for 15 minutes. Then the wells were decanted and stained with crystal violet for 20 minutes. The wells were again decanted and washed with distilled water. Finally 33% glacial acetic acid was added to the wells to extract the stain and adherence of the stained cells to the wells. Optical density of each well was measured at 490 nm using an automated ELISA plate reader [16].

Results

A total number of 150 patients who were on mechanical ventilation for more than 48hrs were included in the study out of which 122 patients were confirmed as VAP according to the Clinical Pulmonary Infection Score. Among which 65.3% were males and 44.7% were females with a mean age of 32.8 ± 12.2 years. The most frequent cause of ICU admission were sepsis followed by suicidal poisoning mainly with organo-phosphorous poisoning the next being closed head injury as a result of road traffic accident (Fig 1). 54.6% of patients who were on ventilation had received prior antibiotic treatment and 45.4% of patients has not received antibiotic before they were put on ventilator. WBC count was raised in all the patients. Out of 150 patients on ventilator, 122 patients' endotracheal samples grew bacteria on culture. 78 endotracheal samples showed growth for mono aetiology and 32 samples showed growth for two bacteria, 12 samples showed poly aetiology. A bacterium which showed the colony count more than 10⁶ CFU/ml were considered as significant pathogen and were further subjected for analysis. The microbial profile on ETT culture is depicted in table 1. Gram negative bacteria were isolated in highest number. *Klebsiella pneumoniae* was the predominant bacteria isolated (28%) followed by *Acinetobacterbaumannii* (24%), *Citrobacterfreundii* (14.9%),

quality control of disc diffusion tests, ATCC control strains of *Escherichiacoli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, and *Pseudomonas aeruginosa* ATCC 27853 strains were used. Biofilm

Table 2: Degree of Biofilm production among the bacterial isolates by Tissue culture plate in Group-I and Group-II

Bacteria isolated	Group I			Group II		
	Strong	Moderate	Weak / none	Strong	Moderate	Weak / none
<i>Klebsiella pneumoniae</i>	11	17	6	4	4	0
<i>Acinetobacterbaumannii</i>	3	4	9	17	6	0
<i>Citrobacterfreundii</i>	4	6	4	2	4	6
<i>Staphylococcus aureus</i>	6	11	2	0	0	0
<i>Pseudomonas aeruginosa</i>	3	6	1	4	0	0
<i>E. coli</i>	2	7	1	0	1	1
<i>Streptococcus pneumoniae</i>	0	1	3	0	2	0
<i>Serratiaspp</i>	0	0	1	0	0	0

Pseudomonas aeruginosa (9.7%) and *E.coli* (6.9%). Among Gram positive bacteria, *Staphylococcus aureus* (11.4%) was isolated in highest number followed by *Streptococcus pneumoniae* (4%) (Table 1). Antimicrobial susceptibility pattern of Gram positive bacteria revealed that *Streptococcus pneumoniae* was sensitive to penicillin and all other antibiotics tested. Among 20 *Staphylococcus aureus* isolated 80% of the strains were resistant to Methicillin. The drug which was most effective against MRSA was Clindamycin, Linezolid and Vancomycin. Antimicrobial susceptibility pattern of Gram negative bacteria revealed that more than 91.9% of the isolates were resistant to at least 3 different groups of antibiotics. Thus majority of Gram negative bacteria isolated were multidrug resistant isolates. Among *Klebsiella pneumoniae* 97.9% of isolates were resistant to Ofloxacin, 89.8% to Meropenem, 83.7% to Imipenem, 79.6% to Ceftazidime +Tazobactam. The most sensitive drug for *Klebsiella pneumoniae* was Amikacin (69.4%) and Cefipime +Sulbactam (61.2%). *Acinetobacter baumannii* was isolated in 24% and all the isolates were resistant to more than 3 classes of antibiotics. 95.2% of isolates were resistant to Ceftazidime, Ceftriaxone, Meropenem and Ceftazidime +Tazobactam, 92.9% of isolates were resistant to Ciprofloxacin, Imipenem, Cefixime and Piperacillin +Tazobactam. None of the isolates were resistant to Colistin. Among *Pseudomonas aeruginosa* 94.1% were resistant to Ciprofloxacin, Cefixime, Ceftazidime, Cefotaxime, 88.2% to Imipenem and Meropenem. The most effective drug of choice in *Pseudomonas aeruginosa* was Amikacin, Cefipime +Sulbactam and Piperacillin +Tazobactam. Out of 148 Gram negative bacteria isolated from VAP, 66.8% were β -lactamase producers out of which 53 were extended spectrum β -lactamase (ES β L) producers, 16 were AmpC, 30 were metallo- β lactamase producers (M β L). *Klebsiella pneumoniae* and *Acinetobacter baumannii* were predominant ES β L producers and *Klebsiella pneumoniae* and *E.coli* was predominant AmpC producers while *Acinetobacter baumannii* and *Klebsiella pneumoniae* were predominant M β L producer (Fig 2 & Fig 3). MDR were common in both the group, but all the bacterial isolates from group II were resistant to multiple commonly used

antibiotics and all the isolates showed the production of biofilm (Table 2). Biofilm production was assessed by tissue culture plate. In Group I patients, 72.4% of the isolates showed either strong / moderate biofilm formation and 21.6% showed either weak / no biofilm production (Table-2). In Group II patients, 92.3 of the isolates showed either strong or moderate biofilm formation and 7.7% showed either weak or no biofilm production.

Discussion

VAP is a common complication of ventilatory support for patients with acute respiratory failure. It is associated with increased morbidity, mortality, and costs. It is important to know the possible organisms causing VAP and specific bacteria in an individual patient to guide optimal antibiotic therapy. Since bacteria causing VAP are known to be multidrug resistant, it is imperative to know the best antibiotic of choice for the treatment. This is probably the single most important management decision in the care of VAP patients because inadequate initial antibiotic therapy leads to excess mortality, and excessive antibiotic therapy increases treatment-related complications and costs and leads to increased prevalence of antibiotic resistance [3,4]. CPIS criteria are important in the diagnosis of VAP. Along with clinical diagnosis, consideration of microbiology of VAP has many additional benefits: It helps to know the prognosis of individual patients, can allow clinicians to track trends in local antimicrobial resistance patterns, can provide insights into the pathogenesis of VAP, can aid the prompt recognition of local VAP outbreaks, and can suggest locally relevant infection control and VAP prevention efforts [17,18]. *K.pneumoniae* was the most common isolate that was identified in the present study. *A.baumannii*, *Citrobacter freundii*, *E. coli*, *P. aeruginosa* and *Serratia* species were the other significant Gram-negative bacteria isolated in the present study. Among the Gram-positive isolates, *S. aureus* was most frequently isolated followed by *Streptococcus pneumoniae*. Causative pathogen of VAP has been known to vary depending on the development time of VAP. In the case of early VAP that occurs within 5 days after mechanical ventilation following intubation, *S. aureus*, *S. pneumoniae*, and *E.coli* are main causative pathogens.

Meanwhile, in the case of late VAP that occurs 5 days or later after mechanical ventilation following intubation, MDR bacteria such as *A. baumannii*, *P. aeruginosa*, and *C. freundii* are the predominant bacteria. Various underlying conditions which necessitate mechanical ventilation and hence, likely to develop VAP are acute respiratory distress syndrome, large-volume lung aspiration, head trauma, and neurosurgery [19]. In the present study, late VAP patients were found associated with various underlying clinical conditions such as sepsis followed by suicidal poisoning mainly by organophosphates and closed head injury after road traffic accident. These findings suggest that intubated patients with any of the associated conditions are at increased risk of pneumonia due to Gram-negative bacteria. VAP due to an MDR microorganism is one of the most dreadful complications that can occur in the critical care setting. Antibiotic selection has the potential to influence the spectrum of bacteria endogenous to the hospital and community [20], and healthcare providers need to appreciate that their antibiotic choices have downstream consequences. Prolonged and indiscriminate use of antibiotics has affected antibiotic resistance patterns and the sensitivities of organisms frequently encountered in the ICU [21]. Another important aspect of the contribution of the ETT in the pathogenesis of VAP is that it serves as a reservoir for microorganisms by providing them a surface to adhere. In other words, it allows the microorganisms to form a biofilm. A biofilm is a permanent source of infection and provides protection to the microorganisms from antibiotics by accretion of the protective glycocalyx [22]. Schuler et al. studied ETT colonization in mechanically ventilated patients and found that all mechanical ventilation tubes had secretions lining the interior of the distal third of the tube that formed a biofilm [22,23]. They noted that it takes 60–96 h to form biofilm after intubation, suggesting the strength of biofilm increases with duration [22]. Even in our study, we observed that 92.3% of isolates were strong or moderate biofilm production in Group II compared to 72.4% among Group I. In biofilms, microbial resistance seems to depend on multiple strategies entirely different from the now-familiar plasmids, transposons, and mutations that confer innate resistance to

individual microorganisms. These include, decreased penetration of the antibiotic into the biofilm, decreased growth rate of the pathogen in the biofilm due to nutrient limitation, quorum sensing among the microbial population of the biofilm. Appropriate antibiotic selection for the treatment of such biofilm associated infections is extremely important. Carbapenems have been the antibiotics of choice for the treatment of infections caused by these organisms, but resistance to carbapenems is becoming common, and very few therapeutic options remain. In our study, 89.1% of the Gram negative isolates were Imipenem resistant. This probably is because carbapenems are the most common antibiotic prescribed by the referring hospital. The potential ability of Gram negative bacteria to form biofilms could explain this outstanding antibiotic resistance [22,23]. In concurrence with the other studies [17,20,21], we noted that 32% of strong biofilm producers also showed nearly complete resistance to all the antibiotics tested, and the resistance was due to ability of the bacteria to produce beta-lactamases enzymes and inability of antibiotics to penetrate the biofilm. Very few studies focus on the prediction of

resistant VAP pathogens. Trouillet et al. evaluated risk factors for infection with potentially drug-resistant pathogens in bronchoscopically confirmed VAP [24]. Overall, potentially drug-resistant isolates were involved in 77 (57%) cases. In our study, multivariate analysis identified three variables independently associated with infection by a potentially drug-resistant pathogen: mechanical ventilation for more than 5 days, prior antibiotic use, and biofilm formation. No potentially drug resistant isolates were identified in the 88.7% of cases of VAP that occurred within the first 5 days of mechanical ventilation in patients who had not received prior antibiotic therapy, whereas potentially drug resistant pathogens such as ESBL, MBL, and AmpC were found in 64.6% of cases diagnosed within 5 days in patients who had received antibiotic treatment. Potentially drug-resistant pathogens accounted for only 26.4% of cases of VAP diagnosed after 5 days of mechanical ventilation in patients who had not received antibiotics. However, when VAP occurred after 5 days of mechanical ventilation in antibiotic-treated patients potentially, drug-resistant isolates were recovered from all the patients. Hence prior antibiotic treatment and prolonged mechanical ventilation are

important risk factors associated with development of multidrug resistance. Since biofilms develop slowly over a period of time, biofilm-related infections are diagnosed in the later course of the disease after the biofilm has been established. Hence treatment in an already biofilm positive case is less effective compared to removing the biofilm present on the ETT. Hence removal of ET tube in regular intervals should be considered with a proper choice of antimicrobial treatment or using ET tube coated with drugs/ biomaterials that discourage biofilm formation may be explored.

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