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Original Research Article

Repercussion of biofilm and antibiotic resistance in ventilator associated pneumonia

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ABSTRACT

Background: Ventilator associated pneumonia contributes nearly half of all cases of hospital-acquired pneumonia. Drug resistance among ventilator associated pneumonia has obligation of device withdrawal in order to achieve clinical and microbiological cure. Aim of the study was to determine the relationship between antibiotic resistance of Endotracheal tube biofilm and pulmonary pathogens in ventilator-associated pneumonia.
Methods: A descriptive analytical study of 100 clinically suspected VAP patients was done. Patients were divided into group-I and Group-II based on intubation duration for 1-5 days and 6-10 days respectively. Endotracheal aspirate (ETA) was collected from clinically diagnosed cases and processed as per standard microbiological techniques. Bacterial counts ≥106 CFU/ml for quantitative cultures was considered significant. Biofilm production was detected by tissue culture plate, tube method and Congo red method. Multi-variant analysis was done to find out the association of the various factors.
Results: *Klebsiella pneumoniae* was the predominant bacteria isolated followed by Acinetobacter baumannii. 45% of Gram negative bacteria were β lactamase producers. In Biofilm production by tissue culture method, 72% of the isolates showed either strong or 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.
Conclusions: Bacterial aetiology, biofilm formation and drug resistance has ramification on outcome of ventilator associated pneumonia. Hence, advised that it is crucial to remove ET tube in regular interval to prevent biofilm formation and sequential cultures to obtain the microbiological information which enables better patient care.

Keywords: Biofilm, Multidrug resistant bacteria, Ventilator associated pneumonia

INTRODUCTION

Ventilator-associated pneumonia (VAP) is the most frequent intensive-care-unit (ICU)- acquired infection, with an incidence ranging from 6 to 52%.[1,2] Several studies have shown that critically ill patient is at high risk for getting such nosocomial infections.[3] The presence of an endotracheal tube (ETT) in ventilated

patients impairs mucociliary clearance and disrupts the cough reflex, thus promoting the accumulation of tracheobronchial secretions and increasing the risk of pneumonia.[4] In addition, the insertion of an ETT could produce injury and inoculate endogenous oropharyngeal bacteria in the low airway tract.[5] Finally, 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. Microorganisms attach to synthetic surfaces, multiply and develop biofilms characterized by the generation of an extracellular polymeric substance or matrix that helps bacteria linger in a favourable microenvironment rather than being swept away by the current.[6] Biofilms have great importance for public health because of their role in certain device related infectious diseases and their role in antibiotic resistance.[7]

Studies have highlighted that isolation of drug resistance among ventilator associated pneumonia has necessity of device withdrawal in order to achieve clinical and microbiological cure. Some data show a good concordance between bacterial colonization of the airway and microbial findings in the biofilm.[8] 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.[9]

Microbial persistence in respiratory airways of pathogens implicated in VAP, even with an appropriate antibiotic therapy, has been related to the lack of response to treatment and to the relapse of VAP.[10] Bacterial survival in ETT biofilm can promote VAP microbial persistence and consequently affect patient prognosis. However, no attempt has been performed in order 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 endotracheal tube biofilm and pulmonary pathogens in ventilator-associated pneumonia.

METHODS

Study type

A descriptive analytical study, with 100 patients admitted to ICU’s of S. S. Institute of Medical Sciences and Research Centre.

Inclusion criteria

Clinically suspected patients according to CDC criteria scored by the Chronic Pulmonary Infection Score (CPIS) were included in the study.[11]

Exclusion criteria

Patients with pneumonia prior to mechanical ventilation or within 48 hours of mechanical ventilation, patients with Adult Respiratory Distress Syndrome (ARDS), cavitary 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, leukopenia <1000cells/mm^3, neutropenia <500PN/mm^3 were excluded from the study.[12,13]

Groups

Patients were divided into group-I and Group-II based on intubation duration for 1-5 days and 6-10 days respectively.

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 endotracheal tube for approximately 24cm.[11-13] The sample was gently aspirated without installing saline and the suction catheters were withdrawn. The sample was transferred into a clean labelled container. The sample was immediately transported to the laboratory for microbiological was performed on all samples before dilutions for estimation of colony count.

Dilution and culture of endotracheal aspirate

ETA was homogenized by vortexing for 1 min followed by centrifugation at 3000rpm for 10 min. One 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 by using Nichrome wire loop with internal diameter of 4mm, which holds 0.01ml of homogenized ETA secretions. Both plates were incubated at 37°C for 16-18 hours. Threshold of bacterial counts ≥106CFU/ml for quantitative cultures from ETA secretions was considered for diagnosis of VAP. Bacterial were identified by standard microbial techniques.[12,14]

Antimicrobial susceptibility testing

The antimicrobial susceptibility testing was performed by Kirby Bauer disc diffusion method according to the criteria put forward by the Clinical Laboratory Standards Institute (CLSI).[15,16] Suspected extended spectrum beta- lactamases (ESBLs) producing organisms were confirmed by double disk synergy test as described previously.[17]

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.[18]

MRSA was detected by using Cefoxitin discs by disc diffusion method. For quality control of disc diffusion tests ATCC control strains of *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923 and *Pseudomonas aeruginosa* ATCC 27853 strains were used.[15-18]

Biofilm production

Biofilm production was detected by three methods, Tissue culture plate method, tube method and Congo red method.[19]

Tissue culture plate method

Overnight culture of the isolate from nutrient agar plate is inoculated into trypticase soy broth (TSB). The primary inoculums are then inoculated in TSB with 1% glucose prepared in different dilutions (1:20, 1:40, 1:80, and 1:100) and loaded into 96 wells flat bottom microtitre plate. Plates are covered and incubated at 37ºC for 24 hours in aerobic condition, the wells are then decanted and washed three times with Phosphate buffer saline (PBS) and fixed with methanol for 15 minutes. Then the wells are decanted and stained with crystal violet for 20 minutes. The wells are again decanted and washed with distilled water. Finally 33% glacial acetic acid is added to the wells to extract the stain and adherence of the stained cells to the wells. Optical density of each well is measured at 490nm using an automated ELISA plate reader.[19]

Tube method

Ten ml of TSB with 1% glucose was inoculated with a loopful of test organism from overnight culture on nutrient agar individually. Broths were incubated at 37ºC for 24 hours. The cultures were decanted and tubes were washed with phosphate buffer saline pH 7.3. The tubes were dried and stained with 0.1% crystal violet. Excess stain was washed with deionized water. Tubes were dried in inverted position and observed for biofilm formation. Biofilm Production was considered positive when a visible film lined the wall and bottom of the tube. Ring formation at the liquid interface was not indicative of biofilm formation. Tubes were examined and amount of biofilm formation was scored as 0-absent, 1-weak, 2-moderate, 3-strong.[19]

Congo Red Agar Method (CRA)

The medium composed of Brain heart infusion broth (37gm/l), sucrose (5gm/l), agar number 1 (10gm/l) and Congo red dye (0.8gm/l). Congo red stain was prepared as concentrated aqueous solution and autoclaved at 121ºC for 15 minutes. Then it was added to autoclaved Brain heart infusion agar with sucrose at 55ºC. Plates were inoculated with test organism and incubated at 37ºC for 24 to 48 hours aerobically. Black colonies with a dry crystalline consistency indicated biofilm production; weak producers usually remained pink, though occasional darkening at the centre of colonies was observed.[19]

RESULTS

Patients were supported with ventilators due to unstable vital signs and or respiratory failure. Characteristics and cause for endotracheal intubation of the patients are shown in the Table 1.

Table 1: Demographic and clinical profile of ventilator associated pneumonia cases among group-I and group-II.

	Total	Group-I	Group-II
Number	100	64	36
Age	34.5±22.8	34.3±3	34.8±8
Sex (M/F)	66/34	45/21	21/13
WBC (X 10^9/l)	10.7±4.9	10.9±4.5	10.6±5.4
RBC (X 10^9/l)	3.7±0.9	3.5±0.7	3.9±1.2
Platelet (X 10^9/l)	1.85±5.7	1.92±5.9	1.83±5.2
Underlying disease			
Sepsis	45	26	19
Poisoning	27	17	10
Closed head injury	15	11	4
Snake bite	9	7	2
Birth asphyxia	2	1	1
Miscellaneous	2	2	-
Prior antibiotic treatment	49	28	21
Patients did not receive antibiotic	51	35	16

Among 100 patients who were on mechanical ventilation for more than 48hrs 66% were males and 34% and the mean age group of the patients was 34.5±22.8 years.

Table 2: Microbial profile of ventilator associated pneumonia in group-I and group-II.

Bacteria isolated	Total	Group-I	Group-II
Klebsiella pneumoniae	29	24	5
Acinetobacter baumannii	27	9	18
Citrobacter freundii	18	10	8
E. coli	10	10	-
Staphylococcus aureus	8	8	-
Pseudomonas aeruginosa	6	2	4
Streptococcus pneumoniae	1	1	-
Serratia spp.	1	1	-

In the present study, 95 endotracheal samples grew bacteria on culture. Among 95 ET samples, 64 showed growth for mono aetiology and 15 samples showed growth for two bacteria, 10 samples showed poly aetiology. A bacterium which showed the colony count more than 10^6 CFU/ml were considered as significant pathogen and were further subjected for analysis. The microbial profile on ETT culture is depicted in Table 2. Gram negative bacteria were isolated in highest number. *Klebsiella pneumoniae* was the predominant bacteria isolated (29%) followed by *Acinetobacter baumannii* (27%).

Antimicrobial susceptibility pattern of Gram negative bacteria revealed that more than 90% of the isolates were resistant to at least 3 different groups of antibiotics.

Among *Klebsiella pneumoniae* 98.3% of isolates were resistant to Ofloxacin, 95% to Meropenem and Ceftazidime + Tazobactam, 96.6% to Ciprofloxacin and Cefixime and 90% to Imipenem. The most sensitive drug for *Klebsiella pneumoniae* was Amikacin and Cefipime +Sulbactum.

Among *Acinetobacter baumannii* 96.2% of isolates were resistant Ceftazidime, Ceftriaxone, Meropenem and Ceftazidime + Tazobactam, 92.3% of isolates were resistant to Ciprofloxacin, Cefixime and Piperacillin + Tazobactam. The drugs of choice for treatment of MDR *Acinetobacter baumannii* in present study are Amikacin, Cefotaxime, Cefaperazone.

Among *Pseudomonas aeruginosa* 94.8% were resistant to Cefixime and Ceftazidime, 92.2% to Cefotaxime, 89.6% to Meropenem and Ceftazidime + Tazobactam. The most effective drug of choice in *Pseudomonas aeruginosa* was Amikacin, Cefipime + Sulbactam and Piperacillin + Tazobactam.

Among *Staphylococcus aureus* isolated 75% of the strains were resistant to Methicillin. The drug which was most effective against MRSA was Clindamycin, Linezolid and Vancomycin.

Among 92 Gram negative bacteria 45% were β lactamase producers out of which 36 were extended spectrum βeta lactamase (ESβL) producers, 14 were AmpC, 33 were metallo-βeta lactamase producers (MβL). *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* were predominant ESβL producers and *Klebsiella pneumoniae* was predominant AmpC producers while *Acinetobacter baumannii* and *Klebsiella pneumoniae* were predominant MβL producers (Table 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 3: Beta-lactamase mediated resistance among gram negative bacilli in group-I and group-II.

Organisms	Group I			Group II		
	ESBL	MBL	AmpC	ESBL	MBL	AmpC
Klebsiella pneumonia	9	4	4	4	3	1
Acinetobacter baumannii	6	4	2	9	14	2
Citrobacter freundii	1	2	1	1	9	1
E. coli	1	2	1	1	1	1
Pseudomonas aeruginosa	2	0	0	3	4	0

Table 4: Biofilm production among the ETT 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
Klebsiella pneumoniae	8	13	3	4	1	0
Acinetobacter baumannii	0	1	8	17	1	0
Citrobacter freundii	2	4	4	2	0	6
E. coli	2	6	1	0	0	1
Staphylococcus aureus	3	3	2	0	0	0
Pseudomonas aeruginosa	0	1	1	4	0	0
Streptococcus pneumoniae	0	0	1	0	0	0
Serratia spp.	0	0	1	0	0	0

Biofilm production was assessed by tissue culture plate, tube method and Congo red agar method. In tissue culture method, 72% of the isolates showed either strong or moderate biofilm formation and 28% showed either weak

or no biofilm production (Table 4). Similarly tube method detected 63% isolates as biofilm producers and 37% as weak or non-biofilm producers. Congo red agar method detected 24% isolates as biofilm producers and 66% as weak or non-biofilm producers.

DISCUSSION

Ventilator-associated pneumonia (VAP) is a common complication of ventilatory support for patients with acute respiratory failure and is associated with increased morbidity, mortality, and costs. First of all, awareness of the potential microbial causes of VAP and confirmation of the specific cause in an individual patient are essential to guide optimal antibiotic therapy. This is arguably the single most important management decision in the care of these 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.[1,2] Attention to the microbiology of VAP has many additional benefits: it may inform 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.[10,20]

In this study, *Klebsiella pneumoniae* was the most common isolate that was identified. The other significant Gram-negative isolates implicated were *Acinetobacter baumannii*, *Citrobacter freundii, E. coli, Pseudomonas aeruginosa* and *Serratia* species. Amongst the Gram-positive isolates *Staphylococcus 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, *Staphylococcus aureus, Streptococcus pneumoniae,* and *E. coli* are main causative pathogens. Meanwhile, in the case of late VAP that occur 5 days or later after mechanical ventilation following intubation, multidrug-resistant bacteria such *Acinetobacter baumannii, Pseudomonas aeruginosa* and *Citrobacter freundii* are the predominant bacteria.

Various risk factors reported for the development of VAP are acute respiratory distress syndrome, large-volume lung aspiration, head trauma and neurosurgery.[21] In the present study, late VAP patients were found associated with various underlying clinical conditions like closed head injury, sepsis, birth asphyxia and chronic obstructive pulmonary disease (Table 1). 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 a 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 and health-care providers need to appreciate that their antibiotic choices have downstream consequences.[22] Prolonged and indiscriminate use of antibiotics has affected antibiotic resistance patterns and the sensitivities of organisms frequently encountered in the ICU. [23]

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. [24] Thus, in biofilms, microbial resistance seems to depend on multicellular strategies entirely different from the now familiar plasmids, transposons, and mutations that confer innate resistance to individual microorganisms. Feltman *et al.*, studied ETT colonization in mechanically ventilated patients and found that all airway access tubes had secretions lining the interior of the distal third of the tube that formed a biofilm.[24,25] They noted that it takes 60-96 hours to form biofilm after intubation suggesting the strength of biofilm increases with duration.[24]

Even in our study we observed that 75% of isolates were strongly positive for biofilm in group II compared to 23% among Group I. Appropriate antibiotic selection for the treatment of such biofilm associated infections is extremely important. Carbapenems have been the antibiotics of choice for treatment of infections caused by these organisms, but resistance to carbapenems is becoming common, and very few therapeutic options remain. In our study, all the isolates were imipenem resistant which is 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.[25,26] In concurrence with the other studies we noted that 53% 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 lactamase enzymes and inability of antibiotics to penetrate the biofilm.[10,22-25] 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.[27] Overall, potentially drug-resistant isolates were involved in 77 (57%) cases. In our study multivariate analysis identified 3 variables independently associated with infection by a potentially drug-resistant pathogen: duration of mechanical ventilation for 5 days, prior antibiotic use, and biofilm formation. No potentially drug-resistant isolates were identified in the 91.4% 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 85.7% cases diagnosed within 5 days in patients who had received antibiotic treatment. Potentially drug resistant pathogens accounted for only 18.8% 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.

CONCLUSION

To conclude bacterial aetiology, biofilm formation and drug resistance has ramification on outcome of ventilator associated pneumonia. Hence, advised that it is crucial to remove ET tube in regular interval to prevent biofilm formation and sequential cultures to obtain the microbiological information which enables better patient care.

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