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Original Research Article

BIOFILM PRODUCTION AND ANTIMICROBIAL RESISTANCE IN VENTILATOR ASSOCIATED PNEUMONIA (VAP) PATHOGENS OF ICUs OF TERTIARY CARE CENTER OF CENTRAL GUJARAT.

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Abstract

Background: In critical care units, Ventilator-associated pneumonia (VAP) is a common device-associated infection in mechanically ventilated patients. Problem gets worst of associated with biofilm producing organism with higher antimicrobial resistance. The current study was carried out to observe the pattern of antimicrobial resistance, biofilm forming capacity of isolates causing Ventilator-associated pneumonia and other risk factors associated with VAP patients in intensive care units of Shree Krishna Hospital, Karamsad.

Methodology: 97 total tracheal aspirate culture isolates recovered from 83 mechanically ventilated patients diagnosed to be suffering from VAP as per NHSN definition, admitted in various ICUs of Shree Krishna Hospital, Karamsad during the study duration were included in the study. Relevant clinical history of the patients and other details taken for various patient variable factors like age, gender, co-morbid conditions, indoor days, ventilator days, final patient outcome and other lab based investigations done as indicator of active pneumonia or sepsis from the electronic hospital database available on hospital information system. The tracheal aspirate culture isolates were then tested for antimicrobial susceptibility testing by Vitek2compact and in-vitro biofilm production assay using microtitre plate method. Objective of the present study was to determine the incidence of antimicrobial resistance, biofilm forming capacity of VAP pathogens, to determine risk factors associated and final outcome in VAP patients infected with biofilm forming pathogens. Chi-square test was used to check the relation between the categorical variables while t test was applied in case of continuous variables. A p value less than 0.05 was considered as statistically significant.

Results: Out of total 83 patients of VAP, 97 isolates recovered in tracheal aspirate culture. Out of total 83 patients, 42 patients (49 isolates) were found Biofilm producer (BFP) and 41 patients (48 isolates) were found Biofilm non-producer (BFNP). Out of 97 culture total isolates, the most common organisms grew were Klebsiella pneumoniae (29 isolates), Acinetobacter baumani (28 isolates) and Pseudomonas aeruginosa (19 isolates) apart from them lesser number of isolates of Staphylococcus aureus (6), Escherichia coli (5), Pantoea spp. (2), Serretia marcescens (2), Pseudomonas putida (1), Sphingomonas paucimobilis (1), Stenotrophomonas maltophila (1), Enterococcus faecium (1), Candida famata (1) and Candida tropicalis (1). The antimicrobial resistance was compared in three major pathogen between BFP and BFNP isolates, i.e. Klebsiella pneumoniae, Acinetobacter baumani and Pseudomonas aeruginosa, which was found to be statistically insignificant. Mortality was recorded higher in BFP patients (16.67%) compared to BFNP patients (7.3%) of VAP, but statistically it was not found to be significant (p value > 0.05).

Conclusions: Incidence of BFP and BFNP associated VAP seen 50.51% and 49.49% respectively out of total 97 isolates. Biofilm forming pathogen causing VAP may not influence the outcome of the patient but, biofilm producer pathogens continue to be associated with pathogens causing VAP in significant amount of total cases. Typical hospital acquired strains like Klebsiella pneumoniae, Acinetobacter baumani and Pseudomonas aeruginosa is recorded frequently compared to other pathogens.

Key words: Intensive Care Unit, anti-microbial resistance, VAP, Bio film, Health care associated infection, Indwelling device associated infection.

Introduction

VAP remains the leading cause of health care associated infection and major challenge for the patient welfare and health care economy for critical care physicians and infection prevention specialists. These infections in developed countries occur in 2–18% of hospitalized patients, with rates of up to 54% in ICUs[1,2]. The incidence rate of VAP is considered to be as high as 13.6/1000 Mechanical Ventilation (MV) days, according to the International Nosocomial Infection Control Consortium of (INICC). VAP mortality rates are estimated at between 15

and 76 percent and increase ICU and hospital stays with a significantly higher cost per patient[3,4].

Infection in VAP develops by direct entry of bacteria to the lower respiratory tract, which may be natural flora of the oropharynx or those present in the hospital via 1) microaspiration, which may occur during intubation itself; 2) production of a biofilm overloaded with bacteria (typically Gram-negative bacteria and fungal species) inside the endotracheal tube; 3) pooling and trickling of secretions around the cuff; and 4) impairment of muco-ciliary clearance[5].

The endotracheal tube (ETT), by impairing muco-ciliary clearance, disrupting the cough reflex and encouraging the accumulation of tracheobronchial secretions in the lung and contaminated oropharynx or gastrointestinal secretions provide a source of VAP pathogens [5,6].

Biofilm is a fine coating of organisms that bind to or contain exopolysaccharides, protecting organisms from antibiotics and the immune system. On the surface of every medical device, bacterial biofilms play a significant role in retaining infectious material and increasing antimicrobial resistance in VAP pathogens which contributes to increased morbidity and mortality[8]. The current study was carried out to observe the pattern of antimicrobial resistance and incidence of biofilm producing pathogens and relationship with other risk factors including final outcome in patients suffering with Ventilator-associated pneumonia in intensive care units of Shree Krishna Hospital, Karamsad.

Materials and methods:

Study type, study setting and study period:

It was an observational study conducted in a tertiary care institution's microbiology department. Isolates of patients declared as Ventilator associated Pneumonia (VAP) between March 2018 and December 2019 were collected. Study was duly approved by Institutional Ethics Committee of Shree Krishna Hospital, Ref no. IEC/HMPCMCE/2015/337/15.

Inclusion criteria:

The isolates from tracheal aspirates identified as pathogens of VAP from cases admitted in various ICUs of Shree Krishna Hospital were included in the present study.

Exclusion criteria:

Patients in whom the tracheal aspirates grew an isolate, that had been previously isolated from the same specimen and that was suggestive of persistent infection were excluded from the present study.

Sample size, Sampling Techniques and Data collection:

Purposive sampling of all patients were included who fulfil the inclusion criteria and fall in the study duration. All clinical isolates recovered from patients of VAP were obtained from diagnostic microbiology lab were preserved for further testing of inviro biofilm production assay. These isolates were processed for antimicrobial susceptibility testing and in-vitro biofilm production assay. Further details of the patients collected included like co-morbid conditions, inpatient-days, ventilator-days, final patient outcome and total WBC count, were collected from the electronic hospital information system.

Protocol for Biofilm production detection using microtiter plate method:

All isolates were screened for their ability to form biofilm by microtiter plate (Flat bottom 96 well sterile microtitre plates, Tarson) method. Study isolates enriched in trypticase soya broth (TSB, Himedia) overnight were taken and the turbidity was adjusted to 0.5 McFarland standard using fresh TSB to adjust cell count between 10^5 -10^6 cells/mL. From the above prepared suspension, aliquots of 100µl were distributed in 96 well microtiter plate containing 100µl of fresh TSB along with negative control. Each isolate and negative controls were tested in triplicate. The plates were incubated for 48 hours at 37°C. After incubation, the content of each tube was aspirated and then washed three times with phosphate buffered saline (PBS) to remove any non – adherent bacteria. 200µl of 99% ethanol was added to each well and kept for 15 minutes to fix biofilm. The wells were decanted, left to dry, and stained with 200µl of 2% crystal violet for another 15 min. Excess stain was rinsed off gently by distilled water or tap water. The plates were air dried. The Optical Density was measured at 570 nm using spectrophotometer (Tulip Lisaquant ELISA reader). Based on the average optical density of three wells, the tested isolates were analysed by ratio of the OD (Ratio = Test isolate OD / negative control OD is calculated). These isolates were classified as, Biofilm non producer (BFNP) with ratio <0.2 and Biofilm producer (BFP) with ratio =/> 0.2. For internal quality control, Biofilm-producing reference strain of *Pseudomonas aeruginosa* (ATCC 27853) and non-biofilm forming reference strains of *Staphylococcus aureus* (ATCC 25923) were used. [14]

Statistical analysis:

Data were entered and analysed with Epi info 7 CDC. Categorical data were expressed in percentages while continuous data were expressed with mean and standard deviation. Chi-square test was used to check the relation between the categorical variables while t test was applied in case of continuous variables. A p value less than 0.05 was considered as statistically significant.

Results:

During the study period, from total 83 patients diagnosed to have VAP, grew 97 isolates in tracheal aspirate culture and all were processed for antimicrobial resistance and its ability to form biofilms, of which 49 (50.51%) were biofilm producer and 48 (49.49%) were biofilm non-producer. The distribution with regards to biofilm producer and non-producer amongst individual genus is described in Table-2.

Table-1 compares patients' characteristics with regards to biofilm producing and biofilm non-producing isolates.

As seen in table-2, the most common isolates were *Klebsiella pneumonia, Acinatobactor baumanii* and *Pseudomonas aeruginosa*. Although the antimicrobial susceptibility pattern was observed and recorded for all 97 isolates, the comparative analysis was done for these three isolates only.

Other isolates reported in less than 6 participants are *Staphylococcus aureus* (6), *Escherichia coli* (5), *Pantoea spp.* (2), *Serretia marcescens* (2), *Pseudomonas putida* (1), *Sphingomonas paucimobilis* (1), *Stenotrophomonas maltophila (1), Enterococcus faecium* (1), *Candida famata* (1) and *Candida tropicalis* (1). % resistance of all gram negative isolates mentioned in table-3, gram positive isolates in table-4 and yeast in table-5.

Table 1: Characteristics of study participants (n=83)

Characteristics	**Biofilm Non producer (BFNP) (%) (n=41)**	**Biofilm producer (BFP) (%) (n=42)**	**p-value**
Mean ventilator days	26.07 ± 19.59	24.52 ± 20.82	0.73
Mean Total WBC count (cumm) at the time of detection of VAP	17051±8341	19921±15829	0.30
Mean Length of hospital stay (days)	31.05 ± 20.24	28.67 ± 19.81	0.59
Mean days to VAP after mechanical ventilation	6.88 ± 6.23	8.071 ±8.75	0.47
Age groups			
Year	07 (17.1%)	07 (16.67%)	0.26
1-10 years	00 (0.0%)	03 (7.14%)	
11-20 years	04 (9.8%)	07 (16.67%)	
21-30 years	03 (7.3%)	08 (191.05%)	
31-40 years	04 (9.8%)	03 (7.14%)	
41-50 years	06 (14.6%)	04 (9.52%)	
51-60 years	08 (19.5%)	03 (7.14%)	
61-70 years	06 (14.6%)	03 (7.14%)	
>70 years	03 (7.3%)	04 (7.14%)	
Sex			
Female	14 (34.1%)	09 (21.43%)	0.19
Male	27 (65.9%)	33 (78.57%)	
Location of admission			
MICU	21 (51.22%)	17 (40.48%)	0.15
PICU	01 (2.45%)	07 (16.67%)	
SICU	16 (39.02%)	14 (33.33%)	
NICU	03(7.31%)	04(9.52%)	
Comorbidities			
Diabetes Mellitus	05 (12.2%)	06 (14.29%)	0.77
Hypertension	07 (17.1%)	10 (23.81%)	0.66
Chronic kidney disease	03 (7.3%)	03 (7.14%)	0.97
Known underlying lung condition	08 (19.5%)	04 (9.52%)	0.32
Patient's outcome			
DAMA	10 (24.4%)	07 (16.67%)	0.24
Death	03 (7.3%)	07 (16.67%)	
Discharge	26 (63.4%)	28 (66.67%)	
Transfer	02 (4.9%)	00 (0.00%)	

Table 2: Isolates among study participants (n=97)

Organism	Biofilm negative (n=48)	%	Biofilm positive (n=49)	%
Acinetobacter baumani	13	27.08	15	30.61
Pseudomonas aeruginosa	6	12.50	13	26.53
Pseudomonas putida	0	0.00	1	2.04
Sphingomonas paucimobilis	1	2.08	0	0.00
Stenotrophomonas maltophila	1	2.08	0	0.00
Klebsiella pneumonia	14	29.17	15	30.61
Escherichia coli	5	10.42	0	0.00
Pantoea sp.	2	4.17	0	0.00
Serratia marcescens	1	2.08	1	2.04
Enterococcus faecium	0	0.00	1	2.04
Staphylococcus aureus	3	6.24	3	6.12
Candida famata	1	2.08	0	0.00
Candida tropicalis	1	2.08	0	0.00

Table 3: Resistant pattern of Gram negative organisms (BFP=Biofilm Producer, BFNP=Biofilm Non-producer, % R=% Resistance)

		Colistin	Tigecyclin	Imipenem	Meropenem	Ertapenem	Doripenem	Amikacin	Gentamicin	Amoxicillin Clavulanic acid	Ampicillin	Cefotaxime	Ceftazidime	Ceftriaxone	Cefuroxime	Cefipime	Cefoperazone-Salbactum	Piperacillin-Tazobactum	Ciprofloxacin	Levofloxacin	Co-trimoxazole
Acinetobacter baumani (BFP)	%R N=15	0.0.	0.0	100	100	-	-	73.3	73.3	-	-	-	-	100	-	100	86.7	100	100	-	100
Acinetobacter baumani (BFNP)	%R N=13	0.0	0.0	92.3	92.3	-	-	53.8	69.2	-	-	-	-	92.3	-	92.3	76.9	92.3	92.3	-	92.3
Pseudomonas aeruginosa (BFP)	%R N=13	0.0	-	38.5	38.5	-	38.5	38.5	38.5	-	-	-	30.8	-	-	38.5	38.5	38.5	38.5	38.5	-
Pseudomonas aeruginosa (BFNP)	%R N=06	0.0	-	16.6	50	-	33.3	33.3	33.3	-	-	-	33.3	-	-	50	50	50	33.3	50	-
Pseudomonas putida (BFP)	%R N=1	0.0	-	100	100	-	100	100	100	-	-	-	100	-	-	100	100	100	100	100	-
Sphingomonas paucimobilis (BFNP)	%R N=1	0.0	-	0.0	0.0	-	0.0	0.0	0.0	-	-	-	-	0.0	-	0.0	0.0	0.0	0.0	-	100
Stenotrophomonas maltophila (BFNP)	%R N=1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.0	0.0
Escherichia coli (BFNP)	%R N=5	0.0	0.0	60.0	60.0	60.0	-	0.0	20.0	80.0	100	100	-	100	100	80.0	60.0	80.0	100	-	100
Klebsiella pneumoniae (BFP)	%R N=15	0.0	6.7	80.0	80.0	73.3	-	80.0	73.3	80.0	100	93.3	-	93.3	93.3	66.7	80.0	80.0	93.3	-	86.7
Klebsiella pneumoniae (BFNP)	%R N=14	0.0	21.4	42.8	50.0	50.0	-	35.7	64.3	64.3	100	78.5	-	78.5	85.7	57.2	42.8	50.0	64.3	-	64.3
Pantoea sp. (BFNP)	%R N=2	50.0	50.0	100	100	100	-	50.0	50.0	100	-	-	-	100	100	100	0.0	100	100	-	100
Serratia marcescens (BFP)	%R N=1	100	-	-	-	-	-	00.	0.0	-	-	100	-	100	-	0.0	100	-	0.0	-	0.0
Serratia marcescens (BFNP)	%R N=1	0.0	-	-	-	-	-	0.0	0.0	-	-	0.0	-	0.0	-	0.0	0.0	-	0.0	-	0.0

Table 4: Resistant pattern of Gram positive organisms (BFP=Biofilm Producer, BFNP=Biofilm Non-producer, % R=% Resistance)

Organisms		Tigecycline	Gentamicin	Ciprofloxacin	Levofloxacin	Co-trimoxazole	Oxacillin	Penicillin G	Clindamycin	Erythromycin	Tetcoplanin	Vancomycin	Linezoild	Tetracycline	High Level Gentamicin	High level Streptomycin
Enterococcus faecium (BFP)	%R N=1	0	-	-	-	-	-	100	-	-	100	100	100	-	100	100
Staphylococcus aureus (BFP)	%R N=3	0	0	100	100	33.33	33.33	100	0	33.33	0	0	0	0	-	-
Staphylococcus aureus (BFNP)	%R N=3	33.33	66.66	66.66	66.66	66.66	33.33	100	66.66	66.66	0	0	0	66.66	-	-

Table 5: Resistant pattern of yeast (BFP=Biofilm Producer, BFNP=Biofilm Non-producer, % R=% Resistance)

Organism		Amphotericin-B	Fluconazole	Flucytosine	Voriconazole	Caspeofungin	Micafungin
Candida famata (BFNP)	**%R N=1**	0	0	0	0	100	100
Candida tropicalis (BFNP)	**%R N=1**	0	0	0	0	0	0

Discussion

VAP is a significant challenge in clinical management of critical care patients, as evidenced in various studies reported across globe ranging from 09 to 78 % with an incidence density range from 3.5 to 46 infections/1000 mechanical ventilation days in Asian countries.[3,4,5] In the present study incidence of VAP is 7.1 per 1000 ventilator days during the specific study period. The incidence density of VAP in a study done by Rajan N et al was 31.7/1000 Ventilator days. The frequency of VAP described in the literature varies greatly, owing to varied level of active surveillance practice, varied level of culture screening of respiratory specimens and a lack of clinical and radiographic criteria with good sensitivity and specificity values for pneumonia diagnosis. According to Gadani et al[10], the incidence rate of VAP was 37% in that study. Deshmukh et al[11] reported a 78 % incidence rate, whereas Rit et al[12] reported a 20 percent incidence rate in Ventilator associated Pneumonia**.**

Role of biofilms in pathogenesis of device associated infection have been described in various studies. Organisms involved in biofilm production possess and operate through quorum sensing and specific genes that are expressed and initiate, establish and regulate functions within biofilm over any surface, differs from genus to genus.[19]

Mechanically ventilated patients are having an endotracheal tube or tracheostomy tube in-situ for prolonged period providing favourable environment for the pathogens to adhere to the artificial surface and establish a biofilm formation. Furthermore, in an ICU environment compromised infection control practices facilitate the entry of pathogen in patient's respiratory tract, providing them an opportunity to adhere to the surface of endotracheal or tracheostomy tube. The biofilms thus formed, serves as a shelter protecting them from immune cells as well as antimicrobial agents as well as provide opportunity to exchange genetic material. The organism from these biofilms are released in to the respiratory tract, triggers an immune response and eventually damages respiratory mucosa causing pneumonia. [19]

In the present study 49 isolates (50.59%) from total 97 VAP isolates were identified as BFP. In a study done by Baidya et al[13] 56.3% isolates from cases of VAP were biofilm producers while 43.7% were biofilm nonproducers. Mulla and Jethwani et al[14] found that 65.4% of the isolates causing VAP were biofilm producers in their study.

In the present study there was no statistical significant difference noted between mean ventilator days and mean length of hospital stay of patients with VAP caused by BFP and BFNP pathogens. We could not find studies comparing mean ventilator days or mean length of hospital stay among the two groups. The duration of ventilation and length of hospital stay may vary and is based on the damage to the lungs after onset of pneumonia and not with the ability of the pathogens to form biofilms.

In the present study there was no statistical significant difference noted for the mean total WBC count on the day of detecting VAP in both BFP and BFNP groups. That signifies the WBC count is not influenced by biofilm formation capacity of organism causing VAP, moreover the establishment of biofilm is actually precedes the onset of pneumonia with BFP organisms.

Amongst both groups of Biofilm producer and non-producer the large number of cases is observed in age group 0-1 year (14) followed by 11-20, 21-30 and 51-60 year (11 each). Here also the prevalence of age groups were statistically insignificant. Male participants are observed more (60, 72%) than females (23, 28%) reported for VAP. In study done by Baidya et al[13] they also reported male to be 69% and females to be 31%.

Pre-morbid illnesses and related co-morbidities may have influenced the increased prevalence of VAP. In present study, overall proportion of diabetes was 13.25% while proportion of hypertension, kidney disease and lung disease were 20.48%, 7.23% and 14.46% respectively. Proportion of Comorbidities between biofilm positive VAP and Biofilm negative VAP participants were statistically insignificant (p value <0.05), probably due to the reason that these patients may acquire infection from health care setup of any type of pathogen, BFP or BFNP and due to relatively weak immune response the organism may colonize and set pneumonia easily.

In present study, overall death was observed in 12.05% of the patients with VAP while proportion of mortality among BFP was 16.67% and 7.3% in BFNP. However, patients' outcomes were statistically not significant between BFP VAP and BFNP VAP patients, may be due to the fact that final outcome is influenced by many factors apart from biofilm producer pathogen.

The statistical analysis of antimicrobial resistance was calculated for three potential pathogens i.e. *Klebsiella pneumonia, Acinetobacter baumani* and *Pseudomonas aeruginosa* individually between BFP and BFNP groups but, no statistically significant difference (p value > 0.05) was recorded in BFP and BFNP. Rest of the 10 strains was identified in less than 6 participants. Similar findings were

seen among Mulla et al[14] that *Acinetobacter spp*. (26.6%) accounted for the maximum cases of VAP with 100% BFP, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* accounted for 21.3% of total VAP cases with 78.3% and 75% BFP. These variations may be due to gradual adaptation of the microbial flora over time as well as difference in local and geographic epidemiology. Moreover, sometimes the infection control measures and infrastructure of the hospital also influences epidemiology of these hospital acquired pathogenic strains.

In present study, *Acinetobactor* strains were resistant to even higher antibiotics like Imipenem and Meropenem (100%) in both BFP and BFNP. While biofilm producer *Klebsiella pneumoniae* were 80% resistant to carbapenem group of antibiotics. Colistin was found having 0% resistance among all isolates of BFP and BFNP in three major pathogens. Similarly, in study done by Mulla et al[13] they found Colistin and Polymyxin B were having 0% resistance in *Acenatobactor spp.*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* strains in both BFP and BFNP groups collectively. In a study done by Baidya S e t al[13], MDR pathogens were found to be prevalent (77.5%) among both biofilm producers and non-producers. Biofilm non-producers made up 35.3 percent of MDR isolates, whereas biofilm producers made up 42.2 percent.

Conclusions: The prevalence of biofilm was found to be 49 (50.60%) out of total 97 isolates obtained from respiratory samples of 83 VAP confirmed cases. MDR organisms were found in case of *Klebsiella pneumoniae*, *Pantoea sp.*, *Pseudomonas putida* and *Acinetobacter baumani*, but Colistin and Tigecyclin are more effective against them than others antibiotics. Various demographic and clinical details of patients were evaluated and compared between biofilm producer and biofilm non-producer group but, they were found not to be influenced by the biofilm producer or non-producer strain in present study, as the difference was not statistically significant (p value > 0.05). The probable reason for the same may be the fact that the patients' conditions, comorbidities and final outcome are multifactorial.

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