

Original Research Article

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Biofilm Production by Organisms Causing Ventilator Associated Pneumonia

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ABSTRACT

Ventilator-associated pneumonia is defined as nosocomial pneumonia occurring more than 48 hours after patients have received mechanical ventilation. VAP in a critically ill patient significantly increases risk of mortality, ventilator time, length of stay and cost of care. The Endotracheal tube is a favourable site for biofilm formation. They are adherent cells which are embedded within a self-produced matrix of This EPS matrix impedes the normal functions of antibodies and the phagocytic cells of the immune system and leads to increased resistance to antibiotics. Aim of this study is to isolate and identify the organisms causing VAP and demonstrate biofilm production. A total of 149 patients were selected for the study. 40.94% developed VAP. The organisms causing VAP was isolated and identified by conventional tests. The organisms were tested for biofilm production by the microtitre plate method using safranin dye and their optical densities measured. The organisms causing VAP were *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus*. 47.73 % were strong biofilm producers, 28.41% were moderate producers, 21.59% were weak producers and 2.27% did not produce any biofilm. Among the 42 isolated which produced strong biofilm, *Acinetobacter baumannii* was the most common organism associated with strong biofilm formation (64.29%) followed by *Klebsiella pneumoniae* (21.43%). The biofilm production by microorganisms is a threat because of its resistance to antimicrobial drugs. This highlights the importance of discovering new strategies focused in the removal of biofilm from the Endotracheal tube (ETT). Well planned and early extubations should be done to prevent biofilm formation on the ETT.

Keywords

Ventilator associated pneumonia, Biofilms, tissue culture plate method, Multidrug resistance

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Introduction

Ventilator associated pneumonia (VAP) has been defined as pneumonia that occurs 48 hours after patients have received mechanical ventilation. Early onset VAP occurs within 4 days of mechanical ventilation and late onset

VAP occurs after 4 days (American Thoracic Society, 2005). It is the second most common nosocomial infection and the most common infection in ventilated patients. The prevalence varies between 9 to 27 % with the maximum risk in the first five days after mechanical ventilation (Chastre and Fagon, 2002;

Atul Ashok Kalanuria *et al.*, 2014). VAP imposes increased length of stay in the intensive care unit and hospital and incurs extra financial burden to the patient (Eagye *et al.*, 2009) (Fig. 2).

Biofilms are aggregates of microorganisms in which the cells are bound by a self-produced extracellular polymeric substance which adhere to a solid surface (Hall-Stoodley *et al.*, 2004). The EPS components comprise extracellular DNA, proteins and polysaccharides (Lear and Lewis, 2012).

They can form on living or non-living surfaces and are due to cellular recognition sites on a surface, nutritional cues and exposure to sub inhibitory concentrations of antibiotics (Karatan and Watnick, 2009). Biofilms can include a single bacterial species or diverse and are protected from the body's immune system and antibiotics (Donlan, 2002).

Endotracheal tubes are used to provide ventilation to patients. They impair mucociliary clearance and facilitate accumulation of tracheo bronchial secretions (Craven and Steger, 1995). The presence of ET tube injures the mucosa and paves the way for the colonization of microorganisms and pneumonia.

It leads to the formation of biofilms which contribute to multi drug resistance (Rello *et al.*, 1996). *Staphylococcus aureus* and *Pseudomonas aeruginosa* are the main organisms involved in biofilm formation although *Acinetobacter*, *Streptococcus* and Gram negative bacteria also can form biofilms (Jamal *et al.*, 2015). There are various methods to detect biofilm production like Tissue Culture Plate (TCP), Tube method (TM), Congo Red Agar method (CRA), bioluminescent assay, piezoelectric sensors and fluorescent microscopic examination (Hassan *et al.*, 2011).

Materials and Methods

This was a prospective study done at the ICU complex of a tertiary care hospital in Chennai for a period of nine months.

Inclusion criteria

Patients who were admitted and underwent mechanical ventilation after two days (age >18 years).

Exclusion criteria

Patients having Respiratory tract infection on admission like Pulmonary tuberculosis, COPD, Asthma and ARDS

A total of 149 patients on ventilator were included in the study. VAP was diagnosed on clinical and microbiological basis using the CPIS (Clinical Pulmonary Infection Score). The endotracheal aspirates were collected and processed with minimal delay. The organisms causing VAP were isolated and were subjected to Antimicrobial susceptibility testing including detection of various resistance mechanisms and screened for their ability to form biofilm by Tissue culture plate / Microtitre plate method.

Microtitre plate method

Colonies of the isolated bacteria causing VAP were grown on nutrient agar and inoculated in Trypticase soy broth (TSB) and incubated at 37°C for 24 hours. The culture was diluted 1:100 with freshly prepared trypticase soy broth.

A 96 well microtitre plate with flat bottom was taken and filled with 230 µl of TSB. First three wells were filled with broth only to check for sterility and non-specific binding. Known positive and negative controls were inoculated in each plate.

Then 20µl of the diluted cultures were added to the wells in triplicate and incubated at 37° C for 24 hours. After incubation the plates were washed with phosphate buffer saline PBS (pH-7.2) to remove the free floating planktonic bacteria. 300 µl of methanol was then added to each well and after 15 minutes, it was discarded and stained using 0.1% safranin dye.

After allowing standing for 20 minutes, the stain was discarded and washed with PBS. The adherent cells are resolubilised with 33% glacial acetic acid.

Optical densities (OD) of the stained adherent bacteria were measured with an ELISA auto reader at a wavelength of 490 nm (Fig. 1). The averages of the three OD values were taken. The OD readings were regarded as an index of bacteria attaching to the surface and forming biofilms. The cut off value of OD (ODc) was calculated (Mathur *et al.*, 2006).

Results and Discussion

A total of 149 patients were included in this study out of which 101 (67.78%) were males and 48 (32.22%) were females. The incidence of VAP in our study was 40.94% %, with 61 of 149 patients developing VAP. The organisms isolated were *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus*. Among the 61 VAP cases, 57.38% were monomicrobial (one bacterial species in tracheal aspirate) and 42.62% were polymicrobial (more than one bacteria in tracheal aspirate) yielding a total of 88 isolates.

47.73 % were strong biofilm producers, 28.41% were moderate producers, 21.59% were weak producers and 2.27% did not produce any biofilm. Among the 42 isolated which produced strong biofilms,

Acinetobacter baumannii was the most common organism associated with strong biofilm formation (64.29%) followed by *Klebsiella pneumoniae* (21.43%).

Among the moderate biofilm producers, 60 % of *Acinetobacter baumannii* and 8 % of *Klebsiella pneumoniae* isolates produced moderate biofilms. Among the weak biofilm producers, 36.84% of *Acinetobacter baumannii* and 31.58% of *Klebsiella pneumoniae* isolates produced weak biofilms.

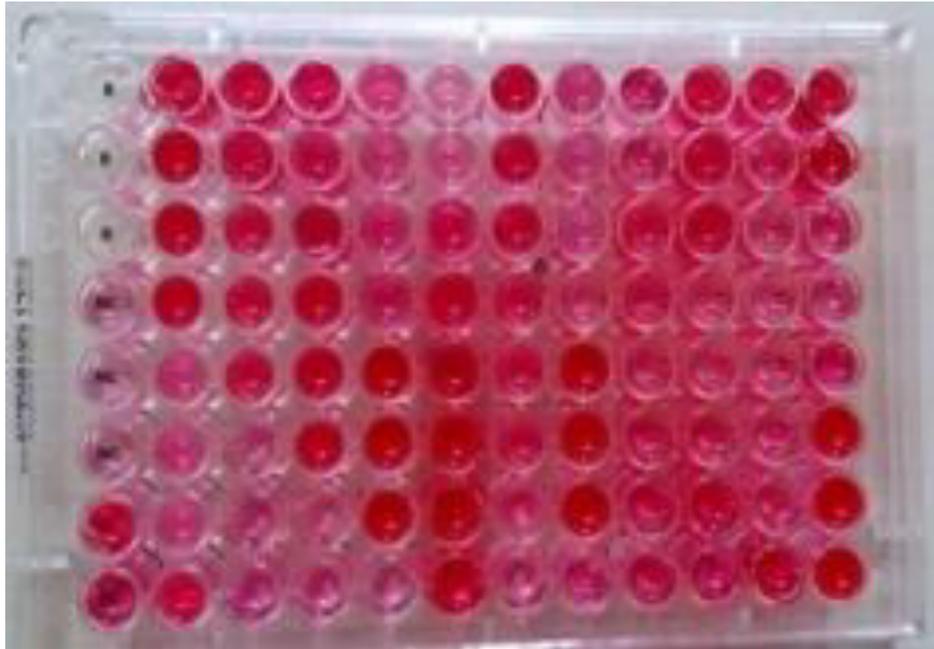
Correlation with multidrug resistance

Multi drug resistance is defined as resistance to three anti-microbial classes. Among the VAP isolates, 69 isolates (78.41%) were multi- drug resistant and 19 (21.59%) were non multi -drug resistant. *Acinetobacter baumannii* produced 54.55 % of multi drug resistance, *Klebsiella pneumoniae* 7.95%, *Pseudomonas aeruginosa* 7.95%, *Staphylococcus aureus* 5.68 and *Escherichia coli* 2.27%.

Isolates with strong adherence in biofilm testing was found to be 36.63% multidrug resistant and isolates with moderate adherence in biofilm testing was found to be 21.59% multi drug resistant. Isolates with weak adherence in biofilm testing was found to be 19.32% multidrug resistant and non-adherent isolates were 1.14% multidrug resistant.

The use of various devices such as intravascular catheters, pacemakers, prosthetic joints has revolutionized the history of medical treatment. But the introduction of these artificial devices in the body has enabled the microorganisms to adhere and form complex biofilms which protect them from the body's immune system and the effect of anti-microbial drugs. Appropriate antibiotic therapy is ineffective in these cases and removal of the device becomes necessary.

Fig.1 Showing microtitre plate with stained organisms



(B-Blank, PC-Positive Control, NC-Negative Control)

Fig.2 Microbiological profile of early and late onset VAP

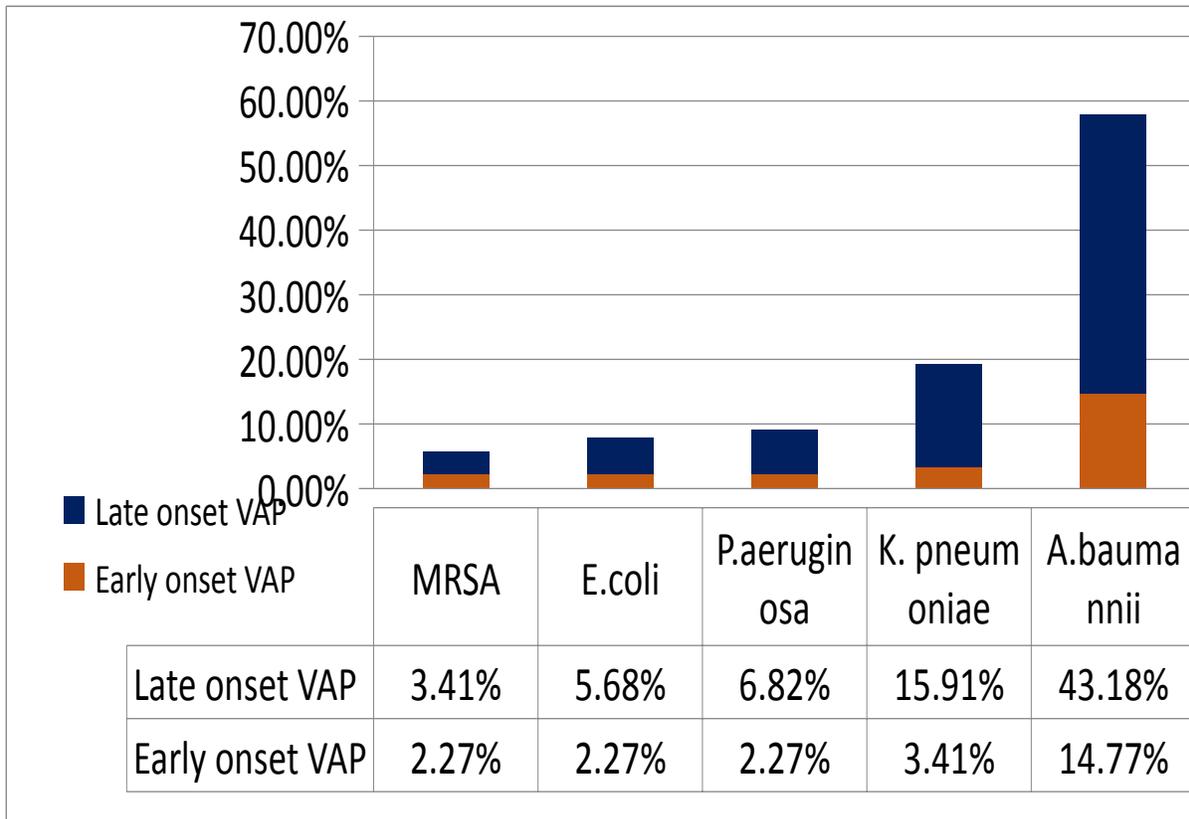


Fig.3 Adhesion profile of biofilm forming microorganisms causing VAP

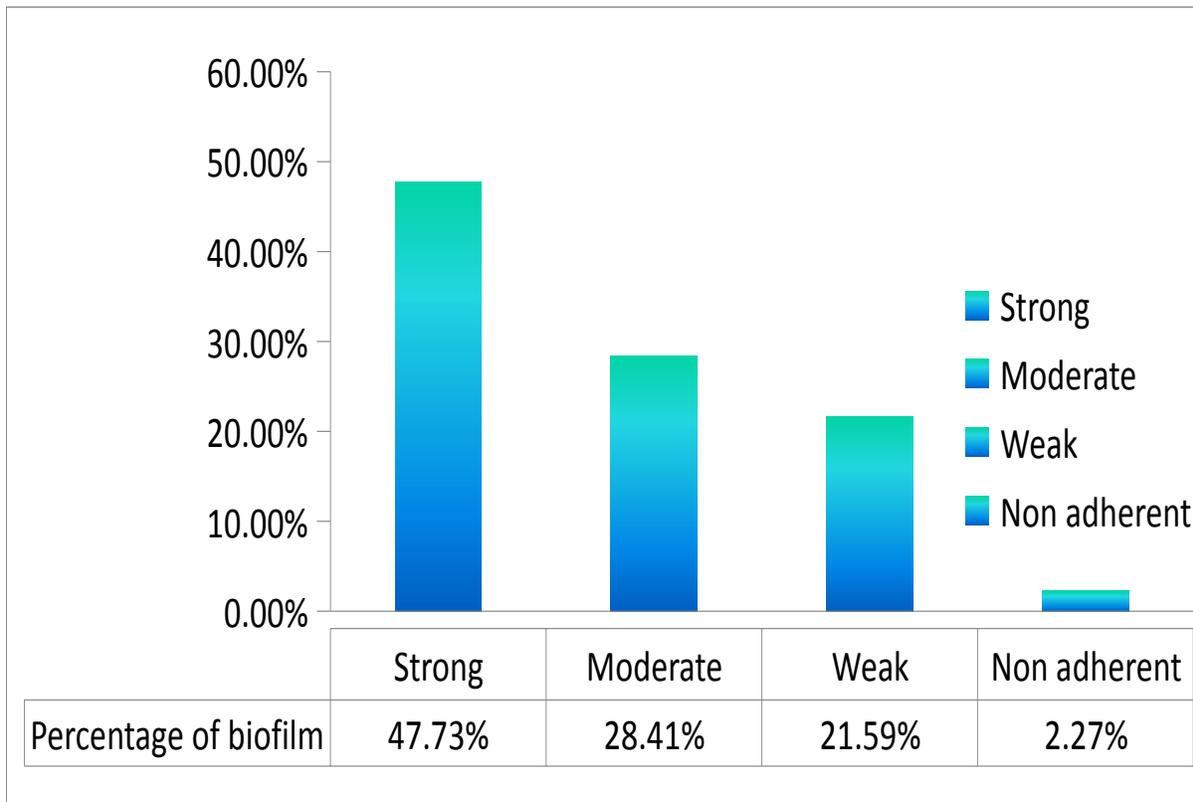


Table.1 Interpretation of biofilm production by microtitre plate method

S. No	OD value	Biofilm
1.	OD < OD _c	Non-adherent
2.	OD _c < OD < 2 OD _c	Weak
3.	2OD _c < OD < 4 OD _c	Moderate
4.	>4 OD _c	Strong

Table.2 Results of biofilm estimation and their association with drug resistance

Organisms	No of organisms	Assessment of biofilms				Drug resistance	
		Strong	Moderate	Weak	None	MDR	Non MDR
<i>Acinetobacter baumannii</i>	51	27	15	7	2	48	3
<i>Klebsiella pneumoniae</i>	17	9	2	6	-	7	10
<i>Escherichia coli</i>	8	3	2	3	-	2	6
<i>Pseudomonas aeruginosa</i>	7	1	4	2	-	7	-
<i>Staphylococcus aureus</i>	5	2	2	1	-	5	-
Total	88	42 (47.73%)	25 (28.41%)	19 (21.59%)	2 (2.27%)	69 (78.41%)	19 (21.59%)

Table.3 Correlation of biofilms adhesion with multidrug resistance

S. NO	ADHESION PROFILE	MDR	NON MDR
1.	Strongly adherent	32(36.36%)	10 (11.36%)
2.	Moderately adherent	19(21.59%)	6 (6.82%)
3.	Weakly adherent	17(19.32%)	2 (2.27%)
4.	Non adherent	1(1.14%)	1 (1.14%)

The diagnosis of device associated infections and the role of biofilms should be considered crucial given its potential to cause life threatening nosocomial infections (Khardori and Yassien, 1995). There are various methods employed for the detection of biofilms such as Tissue Culture Plate, Tube method, Congo red agar method, bioluminescent assays and fluorescent microscopic examination (Hassan *et al.*, 2011). Tissue culture plate method was found to be a better quantitative method when compared to other methods of biofilm detection (Hassan *et al.*, 2011; Lima *et al.*, 2017).

The total number of patients included was 149 out of which 101 (67.78%) were males and 48 (32.22%) were females. The incidence of VAP in our study was 40.94%, with 61 of 149 patients developing VAP.

The organisms causing VAP were *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus*. Among the 61 VAP cases, 57.38% were monomicrobial (one bacterial species in tracheal aspirate) and 42.62% were polymicrobial (more than one bacteria in tracheal aspirate) and the total number of isolates was 88.

Acinetobacter had a propensity to produce 96.08% of biofilm production while the other pathogens *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli* and *Staphylococcus aureus* produced biofilms 100%. Similar studies on biofilm detection

revealed 50% and 62% of biofilm production among *Acinetobacter baumannii* isolates (Gurung *et al.*, 2013; Rao *et al.*, 2008). Another study showed 75 % of biofilm production in *Pseudomonas aeruginosa* (Lima *et al.*, 2017). Mathur *et al.*, in their study found 53.9% biofilm production among *Staphylococcus* spp isolates (Mathur *et al.*, 2006).

47.73 % were strong biofilm producers, 28.41% were moderate producers, 21.59% were weak producers and 2.27% did not produce any biofilm.

Among the 42 isolated which produced strong biofilms, *Acinetobacter baumannii* was the most common organism associated with strong biofilm formation (64.29%) followed by *Klebsiella pneumoniae* (21.43%). Shrinivasa Rao *et al.*, described 62 % of *Acinetobacter* as a strong biofilm producer in their study (Rao *et al.*, 2008).

Among the moderate biofilm producers, 60 % of *Acinetobacter baumannii* and 8 % of *Klebsiella pneumoniae* isolates produced moderate biofilms. Among the weak biofilm producers, 36.84% of *Acinetobacter baumannii* and 31.58% of *Klebsiella pneumoniae* isolates produced weak biofilms.

Correlation with multidrug resistance

Multi drug resistance is defined as resistance to three anti-microbial classes (Magiorakos *et al.*, 2012). Among the VAP isolates, 69 isolates (78.41%) were multi- drug resistant and 19 (21.59%) were non multi -drug

resistant. *Acinetobacter baumannii* produced 54.55 % of multi drug resistance, *Klebsiella pneumoniae* 7.95%, *Pseudomonas aeruginosa* 7.95%, *Staphylococcus aureus* 5.68 and *Escherichia coli* 2.27%. Similar study had a multidrug resistance of 66.1% with *Acinetobacter* as the predominant organism which was similar to our study (Mulla Summaiya and Jethwani Urmi, 2012).

Isolates with strong adherence in biofilm testing was found to be 36.63% multidrug resistant and isolates with moderate adherence in biofilm testing was found to be 21.59% multi drug resistant. Isolates with weak adherence in biofilm testing was found to be 19.32% multidrug resistant and non-adherent isolates were 1.14% multidrug resistant.

The presence of biofilms produced a higher rate of antibiotic resistance among bacteria. *Acinetobacter* had a tendency for strong biofilm production and multi drug resistance. There are no effective treatment strategies for biofilms in VAP patients.

The use of aerosolised antibiotics such as tobramycin, amikacin, colisthmetlate sodium has been found to have a reduction in respiratory infection signs and few studies have found no potential benefit (Palmer and Smaldone, 2014; Russell *et al.*, 2016).

Silver coated endotracheal tube reduces adhesion of bacteria to devices in vitro and hinders with biofilm formation (Kollef *et al.*, 2008; Berra *et al.*, 2004) (Table 1–3 and Fig. 3).

There are many novel treatment options such as phage therapy, quorum sensing inhibition and induced biofilm dispersion. Further research should be done in this field to combat the problem of biofilm induced antibiotic resistance.

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