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ORIGINAL ARTICLE

Scanning Electron Microscopic Biofilm Grading and Ventilator Associated Pneumonia in Relation to Duration of Intubation

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ABSTRACT

Key words:

Ventilator associated pneumonia, ICU, Scanning electron microscopy, MDR bacteria

Background: *Ventilator associated pneumonia (VAP) accounts for 7-70% of infections in intensive care units (ICUs) leading to increase in hospitalization, mortality and cost. Endotracheal tube (ETT) is a risk factor for developing VAP caused by biofilm producing organisms resistant to antimicrobial agents. This study aimed to determine guidelines for ETT extubation through detection of biofilm formation and development of VAP.* ***Objectives:*** *Our aim was to assess biofilm formation on the luminal surface of ETTs in intubated patients in ICU in relation to duration of intubation bacteriologically and morphologically using Scanning Electron Microscope (SEM). Antimicrobial resistance and VAP development were also studied.* ***Methodology:*** *Our work was conducted on 20 ETTs divided into two groups: Group (1); 8 patients with ETTs<5 days, and group (2); 12 patients with ETTs of 5-12 days. Culture of the interior of the ETTs, identification of bacterial species, antibiotic susceptibility and presence with staging of biofilm into 3 grades by SEM were performed.* ***Results:*** *A highly significant relationship was observed between the duration of intubation and biofilm grading (p value <0.001). Also, the duration of intubation and VAP development were significantly correlated (p<0.001). A highly significant relationship was observed between VAP development and biofilm grading by SEM (p<0.001). A moderate significant relationship was found between VAP development and prognosis of patients (p<0.02). It was also observed that prolonged intubation was associated with prevalence of polymicrobial colonization with the predominance of Gram negative bacilli. Furthermore, a moderate significant relationship was observed between antimicrobial resistance and both biofilm grading and duration of intubation (p<0.02).* ***Conclusion:*** *Biofilm formation and grading as well as bacterial colonization with multidrug-resistant (MDR) bacteria were time dependent in patients on mechanical ventilation in ICU which may enhance their morbidity and mortality rates. Also a role of ETT biofilm is emphasized in the pathogenesis and prognostic outcome of VAP in patients intubated for a prolonged period. Accordingly, we recommend that ETT should be exchanged every 4 days to avoid such morbidity and mortality consequences.*

INTRODUCTION

Hospital acquired pneumonia is the most common life-threatening hospital-acquired infection. Majority of cases are associated with mechanical ventilation leading to significant increase in the period of hospitalization, risk of mortality and cost[1]. VAP is a form of nosocomial pneumonia (NP) that develops 48 hours or longer after mechanical ventilation. It is the most common infection in the ICU[2] and the leading cause of morbidity and mortality. The incidence of VAP varies from 7% to 70% and mortality rates from 20 to 75%[3]. Pathogenesis of VAP is multifactorial. Tracheal intubation promotes the aspiration of colonized oropharyngeal secretions across the ETT cuff. It disrupts the cough reflex, promotes accumulation of tracheobroncheal secretions and mucus, and provides a direct conduit for pathogenic microorganisms to reach the lower respiratory tract increasing the risk of infection [4]. The endotracheal tube may also act as a reservoir for pathogens by providing a surface to which they can adhere and form biofilms[5]. The inoculum of pathogens reaches the lungs and cause VAP in critically ill patients with significant impairment of respiratory defenses[6]. Patients who had been intubated for 6 days or longer had a higher percentage of bacterial biofilm compared with patients intubated for less than 6 days[7]. Bacterial biofilms are surface-

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attached communities that are encased in a polymeric matrix, which exhibit a high degree of resistance to antimicrobial agents and host immune system. They contribute in serious diseases such as cystitis, endocarditis, cystic fibrosis, middle-ear and indwelling medical device associated infections [8]. Multiple bacteria are isolated from the interior of the airway access tube of patients undergoing mechanical ventilation in ICU including: *Acinetobacter* spp., *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Proteus* spp., *Escherichia coli*, *Enterobacter* spp., *Citrobacter* spp. and *Staphylococcus* spp.[9]. This study aimed to determine guidelines for endotracheal tube extubation through detection of biofilm formation on endotracheal tubes and development of VAP in ICU at Theodor Bilharz Research Institute (TBRI).

METHODOLOGY

1. Patients:

This was a prospective study done at ICU of TBRI. Twenty patients (15 males and 5 females) aged between 26 and 87 years were mechanically ventilated; 10 were post arrest, 7 post-operative and 3 with chronic renal failure. All intubated patients (males and females ≥ 18 years old) were eligible for the study except for those with acute pneumonia, bronchopneumonia, bronchogenic carcinoma, bronchiectasis, cystic lung or history of previous airway surgery. Permission to undertake investigations was obtained from the Committee for Research on Human Subjects of TBRI with written consent from the relatives of the patients. Full history, clinical examination, laboratory investigations were recorded in each case. The duration of intubation was between 1 and 12 days (mean 5.6 days). All patients received from 2 to 5 antibiotics of the following classes: Beta-Lactams, Beta-Lactam/Beta-lactamase inhibitor combinations, Aminoglycosides, Tetracyclines, Quinolones, Macrolides, Glycopeptides, Fluconazole and Metronidazole.

Diagnosis of VAP was based on the criteria previously described[10]:

1. Develops 48 hours after initiation of mechanical ventilation.
2. New infiltrations on chest X-ray.
3. Worsening gas exchange and at least three of the following criteria: temperature instability with no other recognized cause, new onset of purulent sputum, increase in respiratory secretions or increased need for suctioning, WBC <4000/mm3 or > 15000/mm3, respiratory signs (apnea, tachypnea, nasal flaring, retraction, wheezing, rales, or rhonchi), and bradycardia or tachycardia.

2. Samples:

All samples were taken under complete aseptic conditions and transported immediately to Microbiology and Electron Microscopy Departments for processing. Samples were taken from the upper border of the cuff till the tip, divided longitudinally by sterile surgical scissors. Microscopic samples were immediately fixed in buffered glutaraldehyde[11]. Endotracheal tubes are reinforced Silicone with inner diameter ranging from 7.0 to 8.5 mm. which is the most commonly used in the ICU of TBRI.

3. Processing of Samples:

a. Microbiological study:

Culture of the interior of the endotracheal tubes was done on blood agar, MacConkey's agar, chocolate agar and Sabouraud dextrose agar plates[12]. Identification of bacterial species and antimicrobial susceptibility testing (AST) were assessed by VITEK 2 compact automated system (bioMérieux, France) identifying minimum inhibitory concentrations (MICs) of the following antibiotics: amikacin, ampicillin, ampicillin/sulbactam, cefazolin, cefepim, cefoxitin, ceftazidime, ceftriaxone, ciprofloxacin, gentamicin, imipenem, levofloxacin, meropenem, nitofurantoin, piperacillin/tazobactam, tobramycin and trimethoprim/sulfamethoxazole for Gram negative bacteria , whereas MICs of ampicillin, benzylpenicillin, cefoxitin (screening for MRSA and MRCons), ciprofloxacin, clindamycin, erythromycin, gentamicin, clindamycin, levofloxacin, linezolid, moxifloxacin, nitrofurantoin, oxacillin, quinupristin/ dalfopristin, rifampicin, tetracycline, tigecycline, trimethoprim/sulfamethoxazole and vancomycin were detected for Gram positive bacteria.

Pattern of resistance revealed by AST was interpreted in the form of good sensensitivity showing no resistance to more than 2 classes of antibiotics, multidrug-resistance (MDR) being non-susceptible to at least one agent in three or more antimicrobial categories, extensively drug resistant (XDR) as non-susceptibility to at least one agent in all but two or fewer antimicrobial categories (i.e. bacterial isolates remain susceptible to only one or two categories) and pandrug-resistant (PDR) as non-susceptibility to all agents in all antimicrobial categories.[13]

b. Electron Microscopic study:

Environmental Scanning Electron Microscopic study (Inspect S; FEI, Holland) at Electron Microscopy Unit of TBRI was done on the 20 endotracheal tubes collected from ICU to study the secretions lining the interior of the tubes which is considered the gold standard for morphological study of biofilms[14]. Sections of ETT were processed according to *Glauert*[15]. They were first immediately fixed for 2 hours in equal volumes of glutaraldehyde 4% and caccodylate 0.2 M, washed in equal volumes of sacchrose 0.4 M and caccodylate 0 .2 M for 2 hours and then post fixed in osmium tetroxide 2% and caccodylate 0.3 M for 1 hour. The samples were then washed with distilled water and finally dehydrated in ascending grades of ethyl alcohol for 5 min each (30%.50%.70%.90%) then absolute alcohol 100% for 10 min for 3 times. Specimens were examined with SEM operated at 10–30KV, at the electron microscopy unit of TBRI.

Grading of Biofilm by Electron Microscope:

The biofilm formation was graded using a one to three integer scale[16].

Grade I: shows microcolony formation and the beginning of glycocalyx production.

Grade II: shows an established microcolony, extracellular polymeric substance (EPS) and multidimensional structure and growth.

Grade III: shows a fully mature biofilm covered completely by EPS.

4. Statistical Analysis:

The data were analyzed using statistical package SPSS version 18.0 for windows (SPSS Inc., Chicago, IL). Clinical and laboratory data were shown as mean with 95% confidence interval; a p value of 0.05 was considered statistically significant. All data was presented as mean values or no. of patients. Spearman's rank correlation (r) was used to assess the significant association between continuous variables and microbiology. Diagnostic results between patients were compared using the non-parametric Wilcoxon-Mann-Whitney U-test while Chi-square ($\chi 2$) test was used to compare categorical data.

RESULTS

Patients were classified according to duration of intubation into two groups. Group 1: **(**8 patients) with duration <5 days. **Group 2 (**12 patients) duration ranged from 5-12 days. Bacterial colonization and biofilm formation by SEM were observed on the lumen of 85% (17/20) of the collected tubes while 15% (3/20) showed no bacterial colonization or biofilm formation. As regards staging of biofilm by SEM: 50% (10/20) of the biofilms were grade III (Fig.4,5) ,30% (6/20) were grade II (Fig.3), and 5% (1 /20) was grade I (Fig.1,2). In case of prolonged intubation, macroscopic pillar like structure of mature biofilm was obvious. (Fig 6).

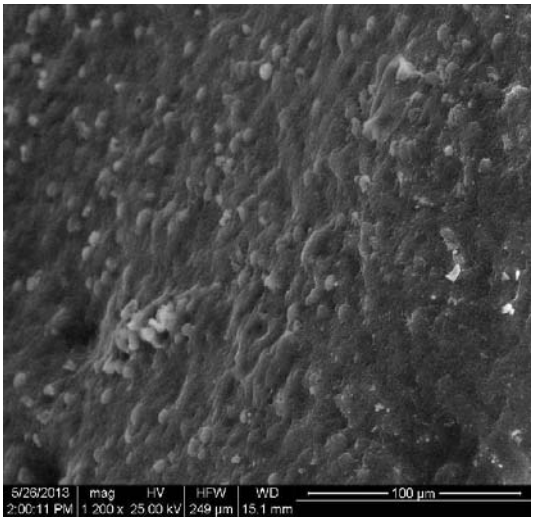

Fig 1: SEM of an ETT showing scattered cocci in a grade 1 bacterial biofilm 1200x. The duration of tube was 2 days.

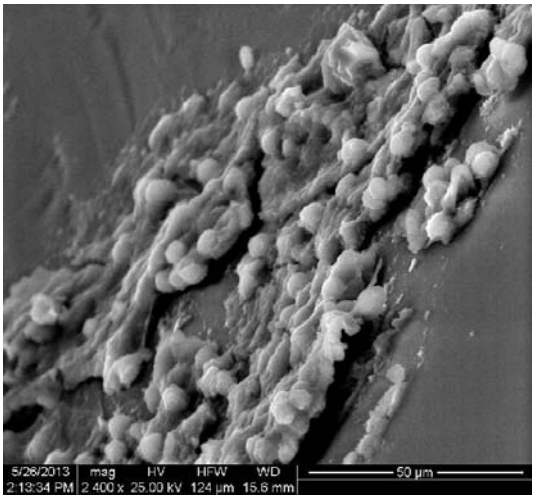

Fig. 2: SEM of an ETT showing microcolony formation and beginning of glycocalyx production in a grade 1 bacterial biofilm 2400x. The duration of tube was 3 days.

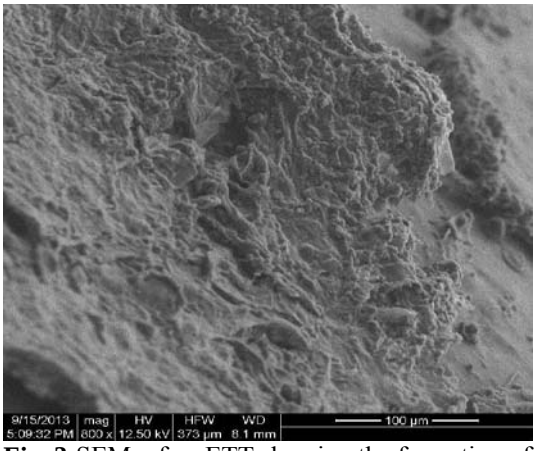

Fig. 3:SEM of an ETT showing the formation of extracellular polymeric substance (EPS) sheets trapping cocci in grade 2 bacterial biofilm 800x. The duration of tube was 3 days.

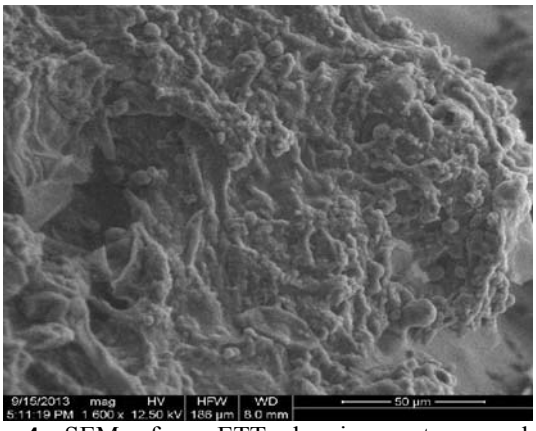

Fig. 4: SEM of an ETT showing mature grade 3 bacterial biofilm 1600x. The duration was 5 days.

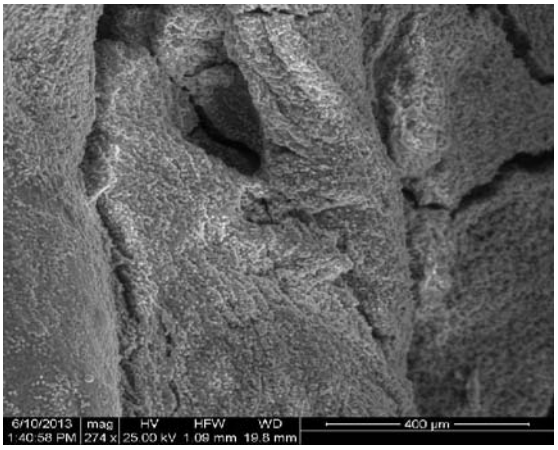

Fig. 5: SEM of an ETT showing fully mature grade 3 bacterial biofilm with open channels between microcolonies resembling primitive circulatory system 274x. The duration of tube was 8 days.

Fig. 6: Part of ETT showing macroscopic pillar like structure of mature biofilm. The duration of tube was 12 days.

Relationship between duration of intubation and biofilm grading by SEM:

In group 1: 37.5% (3/8) showed no bacterial colonization while 50% (4/8) showed biofilm grade II and 12.5% (1/8) was grade I. In group 2: 83.3% (10/12) showed biofilm grade III, while 16.7% (2/12) were grade II. A highly significant relationship was observed between duration of intubation and biofilm grade by SEM ($p<0.001$) as shown in (Fig.7)

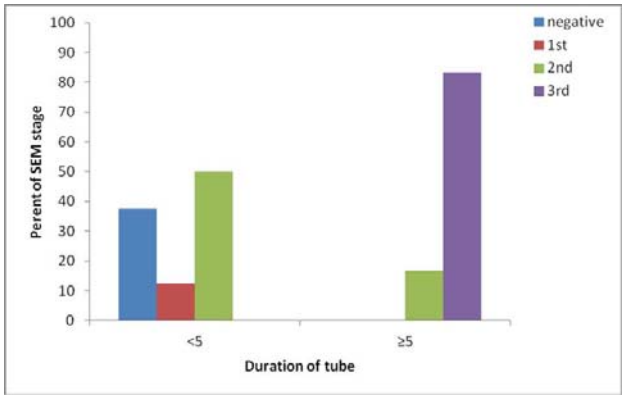

Fig. 7: Relationship between duration of intubation and percent of biofilm grades by SEM.

Relationship between duration of intubation and microbiology:

Inner endotracheal tube surface culture showed the predominant growth of *Acinetobacter baumannii (A. baumannii)* and *Klebsiella pneumoniae (K. pneumoniae)* (each 7/20 cases; 21.2%) followed by *Escherichia coli (E. coli)* (6/20; 18.1%), candida spp. (5/20; 15.2%), *Pseudomonas aeruginosa (P. aeruginosa)* (4/20; 12.1%), *Staphylococcus aureus (S.aureus)* (2/20; 6.1%), coagulase negative Staph (CONS) (2/20; 6.1%). 75% (6/8) of patients in group 1 showed monomicrobial colonization while 25% (2/8) were polymicrobial with predominance of *Staphylococcus* species. Whereas 58.4% (7/12) of patients in group 2 showed polymicrobial colonization with predominance of Gram negative bacilli and 41.6% (5/12) were monomicrobial (Fig.8).

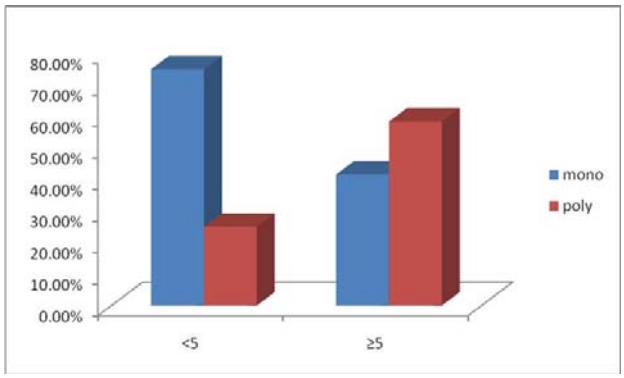

Fig.8: Relationship between duration of intubation and percent of mono & polymicrobial colonization.

Relationship between duration of intubation and AST:

AST of microbiological isolates showed that 10% (2/20) were of good sensitivity, 40% (8/20) were MDR, 30% (6/20) XDR and 20% (4/20) were PDR. In group 1: 25% (2/8) were of good sensitivity, 37.5% (3/8) were MDR and 37.5% (3/8) were PDR isolates, whereas in group 2: 50% (6/12) were XDR, 41.7% (5/12) were MDR and 8.3% (1/12) was PDR. A moderate significant relationship was observed between duration of intubation and AST resistance ($p<0.02$).

Relationship between biofilm grading by SEM and AST:

A moderate significant relationship was also found between biofilm grading by SEM and AST ($p<0.02$). Two (10%) of all patients with isolated pathogens with good sensitivity showed negative biofilm formation, 5 (25%) MDR cases showed grade III biofilm, while 3 (15%) MDR cases showed grade II biofilm, 4 (20%) with XDR isolates showed grade III biofilm while 2 (10%) of XDR isolates showed grade II biofilm and 4 (20%) PDR showed negative, I, II, and III grade biofilm .

Relationship between duration of intubation and VAP:

A very highly significant relationship was observed between duration of intubation and VAP development ($p<0.001$). Eleven out of 20 patients (55%) developed VAP. All of them were in group 2 (91.7%; 11/12) with duration of intubation ≥ 5 days, whereas none of the patients in group 1 developed VAP (Fig.9).

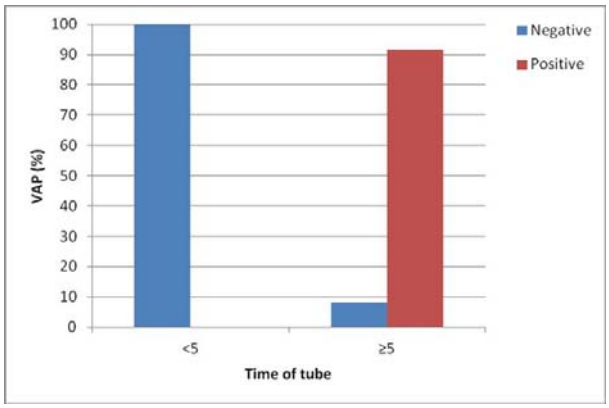

Fig. 9: Relationship between duration of intubation and percent of VAP development.

Relationship between VAP and biofilm grades by SEM:

A very highly significant relationship was observed between VAP development and biofilm grading by SEM ($p<0.001$) (table 3); 3/9 (33.4%) cases who did not develop VAP showed negative biofilm and 1/9 (11.1%) showed grade I and 5/9 (55.5%) cases showed grade II biofilm. Whereas 10/11 (91%) cases who developed VAP showed grade III biofilm while 1/11 (9 %) showed grade II (Fig.10).

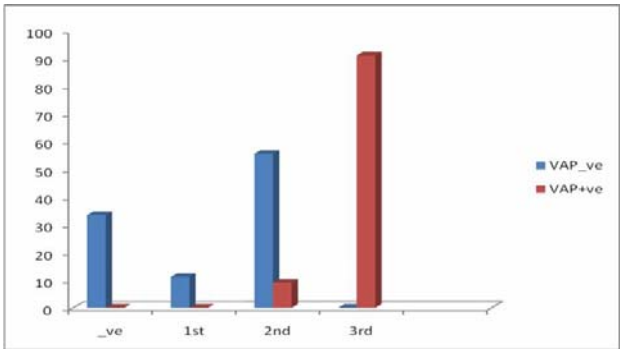

Fig. 10: Relationship between percent of VAP development with biofilm grades by SEM.

Relationship between VAP and prognosis of pateints:

Moderate significant relationship was found between VAP development and prognosis of patients ($p<0.02$); 3/9 (33.3%) cases who did not develop VAP were dead on ventilation while 6/9 (66.7%) were discharged from ICU. Whereas 9/11 (81.8%) with VAP were dead on ventilation and 2/11 (18.2%) cases got better.

DISCUSSION

Colonization of medical devices plays a key role in the problem of healthcare-associated infections[17]. Endotracheal tubes of intubated patients are constantly challenged with abundant bacteria-laden secretions that may organize in a biofilm structure, rendering them difficult to eradicate[18], from which fragments can detach spontaneously or become dislodged by suction catheters and enter the lungs causing infection[19]. In the current study a link has been established between SEM grading of bacterial biofilms on surface of ETTs, microbiological data, antimicrobial resistance pattern and clinical outcome of the intubated patients.

Bacterial biofilm on inner surface of ETTs in intubated patients was revealed in 85% (17/20) of studied cases. This agreed with *Gil-Perotin et al.*[20] who reported even a higher incidence of biofilm 95% (71/75) on ETTs of mechanically ventilated patients. As in a previous report,[7] we have also demonstrated that a longer duration of intubation (more than 6 days) is associated with a higher incidence and grading of bacterial biofilms. Comparing the incidence of colonization among patients at different periods of intubation, we found that bacterial colonization was revealed in 62.5% (5/8) of group 1 (with patients intubated for less than 5 days) and was obvious in 100% (12/12) of group 2 (with intubation duration from 5-12 days). High significant relationship between duration of intubation and biofilm staging (p value <0.001) was observed. In group 1, 50% (4/8) of ETTs showed biofilm grade 2 with established microcolonies, EPS sheets trapping micro-organisms and multidimensional structure and 12.5% (1/8) was grade 1

showing microcolony formation only and the beginning of glycocalyx production. Whereas, in group 2, 83.3% (10/12) showed biofilm grade 3 which appeared fully mature with open channels and cells embedded in glycocalyx forming mushroom-like structures and the remainder 16.7% (2/12) were grade 2. This could be due to the shortage of nutrients as the biofilm gets older giving rise to more compact and mature structure[21].

As previously reported, biofilm formation on the surface of ETTs acts as a likely source of recurrent lower respiratory infections[22]. Our study provided an evidence of the role of ETT biofilm in the pathogenesis of VAP in patients who were intubated and ventilated for a prolonged period confirmed by a high significant relationships between VAP development and biofilm grading by SEM as well as duration of intubation (p value <0.001). 91% (10/11) of the cases with positive VAP showed 3rd grade biofilm while 9% (1/11) showed 2nd grade. Whereas, 55.5% (5/9) of patients who were negative to VAP showed 2nd grade and 33.4% (3/9) showed no evidence of biofilm formation. Also 55% (11/20) of patients who developed VAP were with tube duration of ≥ 5 days, 88.9% (8/9) of patients who did not develop VAP were with tube duration of < 5 days.

Our results agreed with previous study[23] who showed that prolonged intubation period represented one of the risk factors for VAP (P value < 0.001). Many suggested mechanisms explained this correlation such as the detachment of cells from the growing biofilm[24]. However different results were obtained by *Wilson et al.*[25] who didn't find a significant relationship between duration of intubation and development of pneumonia but reported a relationship between advanced biofilm stage and the incidence of pneumonia. This controversy could be attributed to the fact that in this latter study, other risk factors than endotracheal intubation could be responsible for development of pneumonia.

In this study *A. baumannii* and *K. pneumoniae* (21.2% each) followed by *E. coli* (18.1%) were the most common isolated pathogens. Other bacteria including *P. aeruginosa* (12.1%), *Staphylococcus* species (12.2%; 2 CONS and 2 *S. aureus*), as well as candida (15.2 %) were also encountered.

Regarding group 1, 75% (6/8) of ETTs showed monomicrobial colonization and 25% (2/8) were polymicrobial with predominance of *Staphylococcus* species. While in group 2, 58.4% (7/12) of ETTs showed polymicrobial colonization and 41.6% (2/12) were monomicrobial with predominance of Gram negative bacilli. The same results were observed by *Restrepo et al.*[26] who found that the most frequent group of isolated pathogens were Gram-negative bacilli, followed by Gram-positive cocci and by other investigators[27] in Egypt who reported that patients with ETTs were most frequently colonized by *K. pneumoniae* and *A. baumannii* respectively.

MDR bacterial infections, especially those caused by Gram-negative pathogens, have emerged as one of the world's greatest health threats[28]. In this study we demonstrated the drug resistance pattern in relation to the duration and grades of biofilm. The pattern of resistance done using VITEK 2 compact automated system showed moderate significant relationship with biofilm grading by SEM as well as with the duration of intubation (p<0.02). As 10% (2/20) of good sensitivity cases showed negative biofilm formation, 25% (5/20) MDR cases showed 3rd grade biofilm while 15% (3/20) MDR cases showed 2nd grade biofilm, 20% (4/20) XDR cases showed 3rd grade biofilm while 10% (2/20) of XDR cases showed 2nd grade biofilm and 20% (4/20) of PDR showed negative biofilm formation, 1st, 2nd, and 3rd grade biofilm (PDR cases were more related to the type of organism; 2 cases were *A. baumanii*, 1 was *K. pneumoniae* and 1 was *P. aeruginosa*). Same relationship was found regarding duration of intubation, in group 1, 25% (2/8) of ETTs microbiological isolates were of good sensitivity, 37.5% (3/8) were MDR and 37.5% (3/8) were PDR. While in group 2, 50% (6/12) of isolates were XDR, 41.7% (5/12) were MDR and 8.3% (1/12) was PDR. Same results were previously described correlating the ability of biofilm formation of an organism with multidrug resistance[29].

Furthermore we studied the effect of duration of intubation, biofilm grading and VAP development on the prognostic outcome of patients. While we found that only 33.3% (3/9) of cases with negative VAP were dead on ventilation, 81.8% (9/11) of cases with positive VAP were dead. These results showed moderate significant relationship between VAP development and prognosis of patients (p value <0.02). In relation to biofilm grading, we found that one case (1/3) 33.3% with negative biofilm was dead on ventilation but that was due to haematemesis. While 80% (8/10) of cases with grade III biofilm were dead on ventilation. Whereas in relation to the duration of intubation; in group 1 only 37.5% (3/8) of cases were dead on ventilation, while 75% (9/12) of cases in group 2 were dead. This confirms that the longer the duration of intubation, the more patterns of antimicrobial resistance bacteria emerged associated with bad prognostic outcome as previously reported[26].

Hence we found that when intubation duration was ≥5 days, the biofilm became fully mature (grade III) coinciding with the emergence of polymicrobial MDR and XDR isolates and was mostly associated with VAP. We can conclude that ETT colonization and biofilm formation observed in patients undergoing mechanical ventilation are time dependent on duration of intubation and correlated with occurrence of VAP. Bacteria implicated in VAP showed multi resistance towards most antibiotics used in the study. So we recommend that ETT should be exchanged every 4 days to avoid

resistant microorganisms colonization and VAP development.

CONCLUSION

Biofilm formation and grading as well as bacterial colonization with MDR bacteria and VAP were time dependent in patients on mechanical ventilation in ICU which may enhance their morbidity and mortality rates. ETT should be exchanged every 4 days to avoid such morbidity and mortality consequences.

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Conflict of interest:

Authors have no conflict of interests to declare.

REFERENCES

1. Peleg AY and Hooper DC: Hospital-Acquired Infections Due to Gram-Negative Bacteria. New England Journal of Medicine 2010; 362: 1804-1813.
2. Hasan A: Understanding Mechanical Ventilation. In: A Practical Handbook. Hasan A (ed.) Springer Verlag London 2010, pp: 343-389.
3. Emine A and Andreas V: Ventilator associated pneumonia and infection control. BioMed Central 2006; 5: 476-711.
4. Pneumatikos IA, Dragoumanis CK and Bouros DE: Ventilator associated pneumonia or endotracheal tube-associated pneumonia? Anesthesiology 2009; 110: 673–680.
5. Ramirez P, Ferrer M and Torres A: Prevention measures for ventilator-associated pneumonia: a new focus on the endotracheal tube. Curr Opin Infect Dis 2007a; 20: 190–197.
6. Bassi GL and Torres A: Ventilator-associated pneumonia: role of positioning. Current Opinion in Critical Care 2011; 17:57-63.
7. Lee JM, Hashmi N, Bloom JD, Tamashiro E, Doghramji L, Sarani B, Palmer JN, Cohen NA and Mirza N: Biofilm accumulation on endotracheal tubes following prolonged intubation. The Journal of Laryngology & Otology 2012; 126: 267-270.
8. Benoit MR, Conant CG, Zanetti CI, Schwartz M and Matin A: New Device for High-Throughput Viability Screening of Flow Biofilms. Applied and Environmental Microbiology 2010; 76:136–142.
9. Brusselaers N, Vogelaers D and Blot S: The rising problem of antimicrobial resistance in intensive care unit. Ann Intensive Care 2011; 10: 1186-2110.
10. Foglia E, Meier MD and Edward A: Ventilatorassociated pneumonia in neonatal and pediatric care unit patients.ClinMicrobiol Rev 2007; 20: 409-425.
11. Feldman C, Kassel M, Cantrell J, Kaka S, Morar R, GoolamMahomed A and Philips JI: The presence and sequence of endotracheal tube colonization in patients undergoing mechanical ventilation. European spiratory Journal 1999; 13: 546-551.
12. Timothy JJ, Millar MR, Jones G and Robinson DA: Tracheal Tube Biofilm as a Source of Bacterial Colonization of the Lung. Journal of Clinical Microbiology 1989; 27: 2014-2018.
13. Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, Harbarth S, Hindler JF, Kahlmeter G, Olsson-Liljequist B, Paterson DL, Rice LB, Stelling J, Struelens MJ,Vatopoulos A, Weber JT and Monnet DL: Multidrugresistant, extensively drug-resistant and pandrug-resistant bacteria:An international expert proposal for interim standard definitions for acquired resistance. Clin Microbiol Infect 2012; 18: 81-268.
14. Trinidad A, Ibáñez A, Gómez D, García-Berrocal JR and Ramírez-Camacho R: Application of environmental scanning electron microscopy for study of biofilms in medical devices. In Microscopy: Science, Technology, Applications and Education. Méndez-Vilas A and Díaz J (Eds.) 2010, pp: 978-6189.
15. Glauert AM: Fixation, dehydration and embedding of biological specimens. In: Practical Methods in Electron Microscopy. Glauert AM (ed.) Amsterdam Oxford, 1974; pp:77-163.
16. Tatar EC, Unal FO, Tatar I, Celik HH and Gursel B: Investigation of surface changes in different types of ventilation tubes using scanning electron microscopy and correlation of findings with clinical follow-up. International Journal of Pediatric Otorhinolaryngology 2006; 70: 411-417.
17. Talsma S: Biofilms on medical devices. Home Healthcare Nurse 2007; 25: 94-589.
18. Berra L and Kumar A: Prevention of Endotracheal Tube Biofilm Formation in Intubated Critically Ill Patients. Current Respiratory Medicine Reviews 2010; 6: 3-10.
19. Lorente L, Lecuona M, Jimenez A, Mora ML and Sierra A: Influence of an endotracheal tube with polyurethane cuff and subglottic drainage on pneumonia. Am J Respir Crit Care Med 2010; 176: 1079–1083.
20. Gil-Perotin S, Ramirez P, Marti V, Sahuquillo JM, Gonzalez E, Calleja I, Menendez R and Bonastre J: Implications of endotracheal tube biofilm in ventilator-associated pneumonia response: a state of concept. Critical Care 2012; 16: 3-93.
21. Allison DG: The biofilm matrix. Biofouling 2003; 2: 50-139.
22. Chen BM, Yu JL, Liu GX, Hu LY, Li LQ and Li F: Yang H. Electron microscopic analysis of biofilm on tracheal tubes removed from intubated neonates and the relationship between bilofilm and lower

respiratory infection. Zhonghua Er Ke Za Zhi 2007; 9: 60-655.
23. Souza PR, Andrade D, Cabral DB and Watanabe E: Endotracheal tube biofilm and ventilator-associated pneumonia with mechanical ventilation. Wiley 2014; 10: 102-344.
24. Donlan RM: Biofilms: microbial life on surfaces.Emerging Infectious Diseases 2002; 8: 90-181.
25. Wilson A, Gray D, Karakiozis J and Thomas J: Advanced endotracheal tube biofilm. stage, not duration of intubation, is related to pneumonia. Trauma Acute Care Surg 2012; 72: 23-916.
26. Restrepo MI, Peterson J, Fernandez JF, Qin Z, Fisher AC and Nicholson SC: Comparison of the Bacterial Etiology of Early-Onset and Late-Onset Ventilator-Associated Pneumonia. InTech 2013; 58: 1220 1225.
27. Shehata I, Shabban M, Ibrahim R and Shonkry Y: Endotracheal Tube Biofilm and its Relationship to Ventilator Associated Pneumonia in a Neonatal ICU. Nat Sci 2012; 10: 133-140.
28. Flavin MT, Flavin J and Xu ZQ: Combating multidrug-resistant Gram-negative bacterial infections. Expert Opin Investig Drugs 2014; 23: 82-163.
29. Summaiya M and Urmi J: Assessment of biofilm formation by the causative organisms of ventilator associated pneumonia at intensive care unit of a tertiary care hospital. National Journal of Medical Research 2012; 2:15-18.