A study of biofilm on endotracheal tubes in pediatric intensive care unit

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Aim
This study was performed to evaluate the existence of biofilm in the endotracheal tube (ETT) of mechanically ventilated children, and to study a microbial link between biofilm flora and bacteria causing ventilator-associated pneumonia (VAP).

Patients and methods
This was a prospective study conducted on 20 children with ETT who were evaluated for biofilm existence using scanning electron microscopy.

Results
A total of 20 children were enrolled in the study. Of them, 17 (85%) children showed biofilm formation on the luminal surface of ETT. A significant relationship was observed between duration of intubation and biofilm stage. Of the 17 positive cases with biofilm formation, colonization of the inner ETT surface occurred in 14 (70%) cases and 17 isolates were recovered. Five isolates were Gram positive, whereas the majority of isolates were Gram-negative bacilli. Seventeen patients developed pneumonia. All patients who developed biofilm also developed VAP. The occurrence of multidrug resistance among detected microorganisms was high.

Conclusion
We concluded that the density of ETT biofilm increased with increased duration of intubation. We also concluded that ETT colonization with biofilm-producing organisms increased the risk of developing VAP with highly resistant microbes.

Keywords:
biofilm, endotracheal tube, ventilator-associated pneumonia

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Introduction

Ventilator-associated pneumonia (VAP) is one of the most common hospital-acquired infections occurring in mechanically ventilated patients and is associated with increased mortality, longer pediatric intensive care unit (PICU) stay, and health-related costs [1].

The presence of an endotracheal tube (ETT) in ventilated patients breeches natural barriers as it impairs mucociliary clearance and disrupts the cough reflex, which is further hampered by sedation, thus promoting the accumulation of tracheobronchial secretions and increasing the risk for pneumonia [2]. Moreover, ETT facilitates bacterial contamination, through provision of a conduit to the lower airways.

The ETT also acts as a reservoir for pathogens by providing a surface to which they can adhere and form biofilms in just a few hours after its insertion. Biofilm formation occurs when bacteria exude an exopolysaccharide (EPS), causing their aggregation. Bacteria in this type of extracellular matrix form a microenvironment offering them resistance to antibiotics and circumvent the immune response of the patients [3]. Aggregates of ETT biofilm can be easily dislodged by means of suction catheter and disseminate toward the lower respiratory tract [4,5].

Therefore, due to the role of ETTs in the pathogenesis of VAP, some authors suggest that it should be renamed ETT-associated pneumonia [6].

Biofilms have public health significance owing to their role in certain infectious diseases and their role in a variety of device-related infections [7].

The present study used scanning electron microscope (SEM) to detect biofilm formation on the luminal surface of ETTs of mechanically ventilated children in PICU and study its relation with VAP.

Patients and methods

This was a prospective study conducted at the PICU of Cairo University Children Hospital during the period from November 2012 to May 2013.

Twenty children between 1 month and 4 years of age, who were intubated and mechanically ventilated for more than 48 h in the PICU, were included in the study. All patients who developed pneumonia within 48 h of intubation were excluded.

Data collection at the time of admission to the PICU included admission date to PICU, age, sex, underlying diseases, date of endotracheal intubation and mechanical ventilation, surgical procedures, details of antibiotic therapy, steroid usage, and duration of hospital stay.

The diagnosis of VAP in our study was based on the appearance of new or progressive lobar infiltrates more than 48 h after intubation, and two or more of the following minor criteria: fever, leukocytosis/leukopenia, and purulent respiratory secretions confirmed microbiologically [8]. Microbiological criteria included a positive Gram stain (>10 polymorphonuclear cells/low-power field, <10 squamous epithelial cells per low-power field, and >1 bacteria/oil immersion field with or without the presence of intracellular bacteria) and quantitative endotracheal aspirate culture showing more than 10^5 CFU/ml [9,10].

Laboratory investigations included complete blood picture, C-reactive protein, and endotracheal aspirate once VAP is suspected.

The aim and nature of the study was explained to each parent before inclusion. Informed written consent was obtained from parents or caregivers before enrollment. The study design conformed to the requirements of latest revision of Helsinki Declaration of Bioethics (2008). The Scientific Research Committee of Pediatrics Department, Faculty of Medicine, Cairo University, revised and approved the study design.

Collection of endotracheal tubes

The range of tube duration in our patients was not less than 2 days and not more than 10 days, with a mean range of 4.7 days. ETT internal diameter size ranged from 3.5 to 5.5 mm. All ETT tubes were not cuffed. The outer surface of the tubes was cleaned.

A 1-cm cross-sectional segment of the ETT corresponding to the subglottis was cut with a sterile knife. The lowermost 1-cm segment was divided horizontally into two 0.5 cm segments. One half of the segment (0.5 cm) was fixed in 3% glutaraldehyde and cacodylate (readily prepared available from Electron Microscope Unit of Theodor Bilharz Institute) and sent for SEM. The other half was sent for microbiological culture to the microbiology laboratory of the Pediatrics Hospital, Cairo University.

Microbiological study of the endotracheal tube

Swabs of the internal surface of the other 0.5 cm segment were inoculated onto blood, MacConkey's, and chocolate agar media and incubated aerobically at 37°C for 24 h.

Antibiotic susceptibility of microorganisms isolated from quantitative endotracheal aspirate (ETA) cultures was tested using the Kirby–Bauer disk diffusion method, on Mueller–Hinton (Oxoid) agar plates in accordance with the Clinical and Laboratory Standard Institute (CLSI) guidelines [11].

The extended spectrum β-lactamase (ESBL) phenotypic screening and confirmatory tests were carried out according to CLSI guidelines. AmpC β-lactamases were screened using the standard disk diffusion test using 30-μg cefoxitin disks. Isolates with zone diameters of less than 18 mm were considered AmpC positive [12]. Gram-negative bacilli (GNB) were defined as multidrug-resistant (MDR) when they showed resistance to three or more antimicrobial classes [13].

Isolated *Staphylococcus* strains were tested for methicillin resistance using cefoxitin (FOX: 30 μg) for prediction of *mecA* gene-mediated methicillin resistance in the *Staphylococcus* spp., as recommended by CLSI [14].

In addition, *Staphylococcus* strains were tested for resistance using the following disks: penicillin G, cefazolin, amoxicillin/clavulanic acid, ceftazidime, ceftriaxone, cefotaxime, erythromycin, clindamycin, amikacin, gentamicin, ciprofloxacin, doxycycline, vancomycin, and trimethoprim/sulfamethoxazole (Mast Diagnostics, Merseyside, UK).

All media, biochemical reactions, and susceptibility testing were quality controlled using American Type Culture Collection (ATCC) 25922 *Escherichia coli*, *Pseudomonas aeruginosa* ATCC 27853, and *Staphylococcus aureus* ATCC (25923) as reference strains.

Preparation for scanning electron microscopy

The 0.5 cm segment collected from the patients for electron microscopic study was fixed for 2 h in

equal volumes of glutaraldehyde 4% and cacodylate 0.2. It was then washed in equal volumes of saccharose 0.4 and cacodylate 0.2 for 2 h and postfixed in osmium 2% and cacodylate 0.3 for 1 h. Thereafter, it was washed with distilled water and gradually dehydrated in different concentrations of ethyl alcohol for 5 min each (30, 50, 70, and 90%) and then in absolute alcohol 100% for 10 min three times. Specimens were examined with Philips XL30 (Philips, Eindhoven, Netherlands) SEM operated at 10–30 kV, at the Electron Microscopy Unit of Theodor Bilharz Research Institute.

The biofilm formation (its existence and extent on the surface) was reported and graded using a 1–3 integer scale [15].

Grade 1 shows microcolony formation and the beginning of glycocalyx production with few scattered organisms. The beginning of secretion of EPS will attract and coalesce more microbes. Grade 2 shows increased amount of the EPS forming sheets trapping organisms, with large microbial aggregates, established microcolonies, and multidimensional structures and growth. Grade 3 shows a fully mature biofilm as high as a few millimeters pillar-like structures of cells embedded in copious amounts of extracellular polymeric matrix containing thousands of bacteria. Cells embedded in glycocalyx form gross structures resembling towers and mushrooms. Open channels are interspersed between microcolonies resembling the primitive circulatory system [16,17].

Statistical methods

Statistical analysis was performed using SPSS-17 software (SPSS Inc., Chicago, Illinois, USA). The following tests were used: frequency distributions, percentage distributions, mean ± SD, Spearman's coefficients for correlations, and Cohen's κ test for inter-rater agreement. *P*-values less than 0.05 were considered significant.

Results

ETTs were retrieved from 20 mechanically ventilated children (12 boys and 8 girls) between 1 month and 4 years of age. Clinical characteristics of the patients are tabulated in Table 1.

The duration of intubation before ETT collection was between 2 and 10 days (mean 5.15 days).

Duration of intubation was as follows: three cases were intubated for 10 days, one case for 8 days, two cases for 7 days, four cases for 5 days, four cases for 4 days, two cases for 3 days, and three cases for 2 days. All patients received antibiotics.

Table 1 Clinical characteristics of patients

Variables	*N* (%) (*N* = 20)
Age (mean ± SD) (months)	12.75 ± 16.6
Sex	
Female	8 (40)
Male	12 (60)
Antibiotic intakes	20 (100)
Antacid intake	5 (25)
PRISM III score (mean ± SD)	7 ± 6.5
Cause of intubation	
Cardiac	10 (50)
Neurologic	6 (30)
Respiratory	2 (10)
Sepsis	2 (10)

Results of scanning electron microscope

Seventeen (85%) tubes showed biofilm formation on luminal surfaces of ETT, whereas three (15%) tubes showed no biofilm formation. Two (11.7%) cases were of grade 1, four (23.5%) cases were of grade 2, and 11 (64.7%) cases were of grade 3 (Figs. 1–4).

A statistically significant correlation was observed between duration of intubation and biofilm stage ($P = 0.0005$).

Correlation of biofilm formation with other factors such as sex, antibiotic therapy, and antacid administration was not statistically significant ($P > 0.05$).

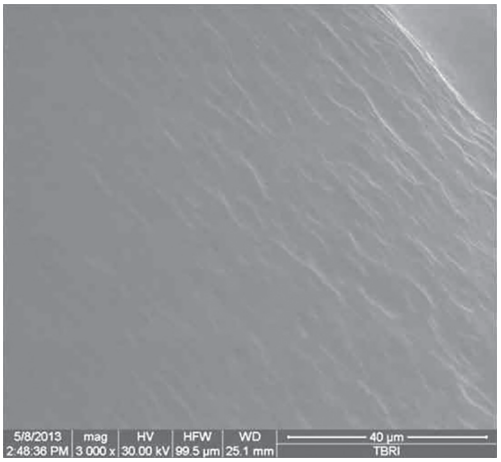
Results of endotracheal tube culture

Of the 17 positive cases with biofilm formation, colonization of the inner ETT surface was detected in 14 (70%) cases, and 17 isolates were recovered (11 patients revealed a single isolate and three patients revealed two isolates). Five isolates were Gram positive, coagulase-negative *Staphylococcus* (CONS) (29.4%), whereas the majority of isolates were GNB: *Klebsiella* spp., six (35.3%) isolates; *Acinetobacter* spp., four (23.5%) isolates; *Pseudomonas* spp., one (5.88%) isolate; and *Proteus* spp., one (5.88%) isolate.

Results of quantitative ETA culture

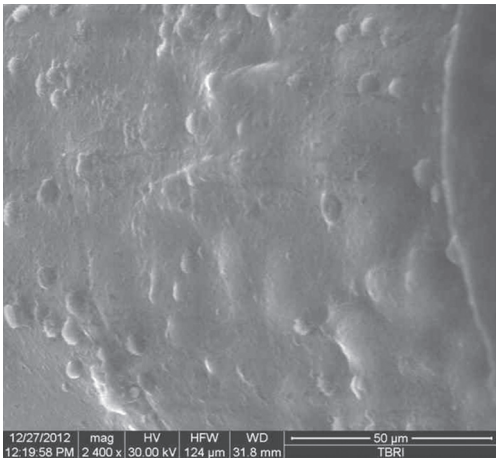
Seventeen (85%) patients fulfilled the criteria for the diagnosis of VAP. These 17 (85%) children were confirmed by positive endotracheal aspirate cultures. A total of 22 isolates were recovered: 12 (70.6%) patients revealed a single isolate and five (29.4%) patients revealed two isolates. The diversity of microbes isolated

Figure 1



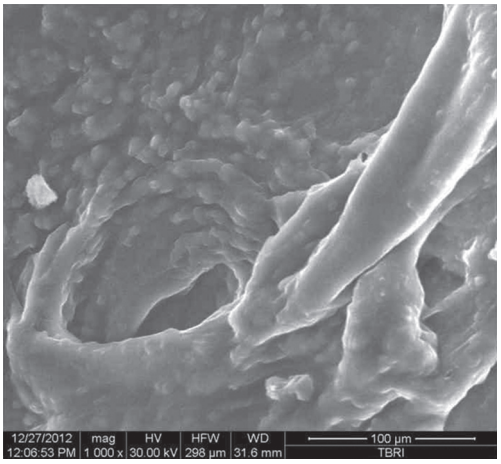
Scanning electron micrograph of an endotracheal tube with no evidence of bacterial biofilm (×3000). The duration of intubation was 48 h.

Figure 2



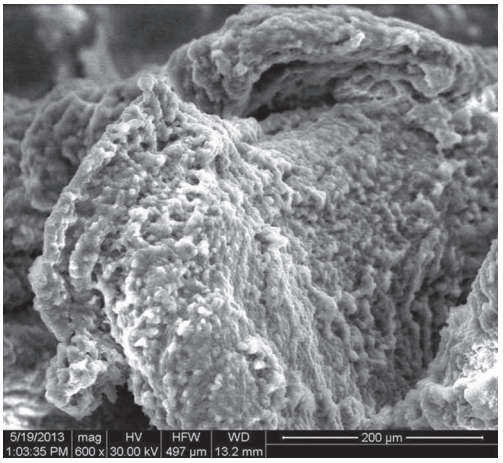
Scanning electron micrograph of an endotracheal tube at higher magnification showing (grade 1) bacterial biofilm (×2400) with scattered large cocci. The duration of intubation was 2 days.

Figure 3



Scanning electron micrograph of an endotracheal tube showing (grade 2) biofilm (×600) with sheets of exopolysaccharide and a number of trapped cocci. The duration of intubation was 3 days.

Figure 4



Scanning electron microscope of an endotracheal tube showing (grade 3) mixed biofilm with homogenous exopolysaccharide completely surrounding the cocci and trapping some bacilli over its surface (×600). Cells embedded in glycocalyx forming gross structures resembling towers and mushrooms; the duration of intubation was 9 days.

from quantitative ETA cultures was as follows: eight (36.36%) *Klebsiella* isolates, four (18.18%) *Acinetobacter* isolates, three (13.6%) *Pseudomonas* isolates, and two (9.1%) isolates of *Proteus*, in addition to five CONS isolates (22.7%).

No fungal infection was reported during this study.

Relationship between biofilm formation and ventilator-associated pneumonia

All patients who developed biofilm also developed VAP, and in the three (15%) children who had negative endotracheal aspirate culture results no biofilm was observed on their ETTs by means of SEM. This was translated statistically by a 100% agreement between biofilm formation and ETA culture results (Cohen's $\kappa = 1$).

Relationship between pathogen on endotracheal tube and aspirate culture

Among the VAP-positive patients, nine samples had the same pathogen both on the inner surface of ETTs and in the endotracheal aspirate, which accounted for 52% of the positive cultures from ETTs. Five (29.4%) samples of ETA cultures showed one organism phenotypically similar to ETT inner surface culture. Three samples gave positive ETA culture but negative ETT culture.

Results of antimicrobial susceptibility testing

Among CONS isolates, methicillin resistance was detected in 60% of isolates. Results of sensitivity testing to the other antibiotic disks were as follows: vancomycin, 100%; doxycycline, 100%; trimethoprim sulfamethoxazole, 60%; ciprofloxacin, 40%; amikacin, 20%; penicillin, 0%; erythromycin, 0%; and clindamycin, 0%.

For the Gram-negative isolates, susceptibility was as follows: carbapenems, 41.1%; ciprofloxacin, 64.7%; trimethoprim sulfamethoxazole, 29.4%; gentamicin, 35.2%; amikacin, 17.6%; cefoxitin, 11.7%; however, susceptibility to ampicillin–sulbactam, cefepime, cefotaxime, ceftriaxone, ceftazidime, piperacillin, piperacillin tazobactam, and cefoperazone/sulbactam was 0%. For the GNB, ESBL production was detected in 100%, whereas AmpC production was detected in 88.2%. Among the isolates of GNB, 70.5% were MDR. MDR was found in 75% of *Acinetobacter*, 66.6% of *Pseudomonas*, 62.5% of *Klebsiella*, and 100% of *Proteus* isolates.

Discussion

Biofilm is considered the new paradigm of infection, especially associated with implants of prostheses, tubes, or catheters, such as the ETT. It has been estimated that up to 80% of all infections worldwide are biofilm related [18].

The presence of the ETT undoubtedly favors microbial colonization, thus contributing to the development of biofilm, which may explain the occurrence of pneumonia.

In this study, biofilm formation was observed on 85% (17/20) of inner surfaces of collected ETTs using SEM. These results are in accordance with previous studies that reported a high prevalence of biofilm formation on ETT [12,19–21], in which biofilm formation was observed on 80–100% on the inner surfaces of collected ETTs using SEM.

The temporal relation between biofilm stage and intubation duration was significantly observed in this study, in which higher staging of biofilm was related to increase in the duration of intubation ($P = 0.0005$).

Our findings are in accordance with those of another study [22]. They used SEM to investigate microbial biofilms on luminal surfaces of ETTs removed from neonates. They found that biofilm architecture became more mature and complex if the duration exceeded 3 days.

In contrast with our study, Wilson *et al.* [23] performed a study on 32 adults and found that the duration of intubation had no relationship with biofilm stage.

The microbial link between biofilm formation and VAP development was very prominent in this study. The diagnosis of VAP was clinically and microbiologically (by positive ETA cultures) confirmed in all patients with positive biofilm formation detected by means of SEM. This was translated statistically by 100% agreement between biofilm formation observed by means of SEM and VAP development ($\kappa = 1$).

Similar results were observed in another study by Adair *et al.* [24], who observed that 70% of patients with VAP had identical pathogens from both ET biofilm and ETA and that these microbes exhibit significant antibiotic resistance.

The types of causative microorganisms depend mainly on the duration of mechanical ventilation and prior antibiotic use, particularly broad-spectrum antibiotics. After prolonged mechanical ventilation and the extensive use of antibiotics, potentially drug-resistant bacteria become prominent causes of VAP and are associated with significantly poorer prognoses [25].

The ESKAPE pathogens (i.e. *Enterococcus faecium*, *S. aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *P. aeruginosa*, and *Enterobacter* spp.) play a crucial role in the development of VAP and were reported to reach 80% of all VAP episodes [26]. These organisms survive unfavorable hospital environments, such as desiccation, nutritional starvation, and antibiotic treatment; it is postulated that their persistence stems from their capacity to colonize medical devices.

In the current study, the authors found that the most prevalent isolates in quantitative ETA cultures were GNBs (77.3%) such as *Klebsiella*, *Acinetobacter*, *Pseudomonas*, and *Proteus* spp., whereas Gram-positive organisms accounted for 22.7% represented by CONS.

Ying *et al.* [27] detected that the majority (77%) of isolated pathogens from neonatal VAP were GNB and the most frequently isolated organisms were *Klebsiella* (20%), *Stenotrophomonas maltophilia* (18%), and *Acinetobacter* (13%).

CONS constituted 22.7% of all isolates obtained from ETA cultures from VAP patients, and they were the sole agent in 80%. Although the exact role of CONS in the pathogenesis of VAP is not well known, several studies demonstrated the presence of CONS in ETT biofilms [5].

Microbial match between ETT biofilm culture and quantitative ETA culture was high, with concordance of nine (52%) samples: five samples showed phenotypic resemblance and three cases showed negative ETT culture.

This finding is in accordance with the results of Gil-Perotin *et al.* [19], who stated that, in 56% of cases, the same organisms could be identified in ETA and ETT cultures.

In the current study, the frequency of polymicrobial infection was present in five (29.4%) VAP patients.

Several researchers estimated that polymicrobial infections accounted for 20–60% of VAP [23].

Increased antibiotic resistance of ETT biofilm-forming organisms is a result of the combination of various factors, such as the selection of more resistant microorganisms, physiological adaptations such as the low rate of multiplication, and the production of EPS [28].

In the current study, antimicrobial resistance rate was alarming in the clinical isolates obtained from VAP patients. The organisms detected in the present study were highly resistant strains; MDR GNB represented 70.5% of the studied isolates. Moreover, ESBL and AmpC production were detected in 100 and 88.2% of GNB, respectively. Among isolates of CONS, methicillin resistance was detected in 60% of cases.

Summaiya and Urmi [29] estimated that 66% of the isolates in their study were MDR, with *A. baumannii* showing the higher rates of MDR, followed by *Klebsiella* spp., *E. coli*, and *S. aureus*.

Chun *et al.* [30] also reported a high rate of MDR in biofilm-associated infections, particularly among *P. aeruginosa*, *K. pneumoniae*, *Enterobacter*, and *Serratia* isolates.

Our study supports the claim that it is ETT-associated pneumonia rather than VAP that causes the major concern. It paves the way for other studies comparing the efficacy of different materials of ETTs to prevent biofilm adhesions, thus preventing VAP. The small sample size is the main limitation of the study.

Conclusion

We concluded that the density of ETT biofilm increased with increased duration of intubation. We also concluded that ETT colonization with biofilm-producing organisms increased the risk of developing VAP with highly resistant microbes.

Acknowledgements

K.S. and S.A. conceived the study, and participated in its concept, design and definition of intellectual content. S.A. performed the clinical part of the study, and prepared the manuscript. M.B. carried manuscript preparation editing and review. Y.S. carried out the processing of the ETT for preparation for electron microscopy and literature research. K.I.D. carried out the microbiological analysis of the samples and helped in data acquisition and analysis. M.M. performed the work of SEM and statistical analysis. All authors read and approved the final manuscript.

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Conflicts of interest

There are no conflicts of interest.

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