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Bacterial profile and biofilm intensity on endotracheal tubes following chlorhexidine use in critically ill patients

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Abstract

***Objective*: To identify bacterial species colonizing the endotracheal tube (ETT) and to evaluate the extent of biofilm formation in patients undergoing oral hygiene protocols with 0.2% chlorhexidine. In addition, the study aimed to determine the correlation between the duration of ETT placement and the degree of bacterial biofilm formation.**

***Design*: Observational analytic study.**

***Setting*: Intensive Care Unit (ICU) of Dr. Soetomo General Hospital, Surabaya, Indonesia.**

***Patients and participants*: Endotracheal tubes were collected from 41 mechanically ventilated ICU patients who had undergone routine oral hygiene with 0.2% chlorhexidine. Patients were selected consecutively, and all ETTs were processed for microbiological analysis with the roll-plate culture method and the enzyme-linked immunosorbent assay (ELISA) biofilm microplate reader.**

***Interventions*: There was no additional therapeutic intervention beyond standard ICU care, including ventilator-associated pneumonia (VAP) bundles and chlorhexidine-based oral hygiene every 8 hours.**

***Measurements and results*: A total of 67 bacterial isolates were obtained from the oral-exposed surface of ETTs. Among these, 85% (59 isolates) demonstrated biofilm-forming capability, with Klebsiella pneumoniae (33%), Pseudomonas aeruginosa (21%), and Acinetobacter baumannii (10%) being the most common species. Gram-negative bacteria formed the majority of biofilm-positive isolates and exhibited stronger biofilm intensity compared to Gram-positive bacteria. No statistically significant correlation was observed between ETT usage duration and biofilm intensity ($p>0.05$).**

***Conclusions*: High rates of biofilm formation were identified on the outer surfaces of ETTs in ICU patients despite routine chlorhexidine oral care. No significant association was observed between the duration of ETT use and biofilm intensity. These findings highlight the need for additional preventive strategies beyond chlorhexidine to reduce the risk of biofilm-associated complications in mechanically ventilated patients.**

Keywords: Biofilm, endotracheal tube, chlorhexidine, critically ill patients, bacterial colonization.

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Introduction

Microorganisms on medical devices, such as endotracheal tubes (ETTs), frequently develop biofilms as a survival strategy, potentially serving as a source of pathogens over time. Biofilms that develop on ETTs are probably significant microbial reservoirs and are associated with nosocomial respiratory infections in mechanically ventilated patients (ventilator-associated pneumonia [VAP]). (1) Our study identified biofilms in 72 out of 75 ETTs from mechanically ventilated patients. In 50% of VAP cases, the same pathogens were identified in both the pulmonary fluid samples and the biofilm within the ETTs, correlating with treatment failure. (2)

The implementation of a ventilator bundle in patient care has demonstrated a reduction in the risk of micro-aspirates, bacterial colonization, and infection; yet, it does not entirely eradicate the pathological process. (3) The use of the VAP bundle aims to inhibit the growth of intraoral pathogenic microorganisms, hence diminishing microbial colonization on both the oral mucosa and the surfaces of medical devices within the oral cavity. This bundle encompasses oral hygiene care (OHC) with a 0.2% chlorhexidine solution. (4) The antibacterial mechanism of chlorhexidine is described by disrupting membrane integrity and enzyme function in the cell membrane, which leads to cytoplasmic leakage, coagulation, and precipitation of intracellular components. However, the effectiveness of chlorhexidine in preventing biofilm formation remains controversial, especially on the medical device surfaces in critically ill patients who are at higher risk of infection. There has also been increasing evidence of microbial resistance to this chemical in recent decades. (5-7)

This study sought to identify bacterial species colonizing the ETT and to evaluate the extent of biofilm formation in patients undergoing oral hygiene protocols with 0.2% chlorhexidine. In addition, the study aimed to determine the correlation between the duration of ETT placement and the degree of bacterial biofilm formation.

Materials and methods

This research was performed at the Microbiology Laboratory of the Integrated Diagnostic Center at Dr. Soetomo General Hospital, Surabaya, Indonesia, and at the Integrated Medical Laboratory of the Faculty of Medicine, Universitas Airlangga, Surabaya.

The clinical sampling process occurred over a duration of one month. We closely monitored the implementation of VAP bundles, which included OHC in accordance with the present method using 0.2% chlorhexidine solution every 8 hours. The sample population was ETT taken from adult patients aged 18 years and older who had been extubated, underwent ETT replacement with tracheotomy, or died. Other criteria were patients who did not experience facial bone fractures or oral cavity infections and were using mechanical ventilation in the Intensive Care Unit (ICU) of Dr. Soetomo Hospital for 2–14 x 24 hours post-intubation, which was also conducted at Dr. Soetomo Hospital. Following extubation, the ETT was cut in a sterile manner, commencing 1 cm above the cuff and extending 5 cm cranially, to delineate solely the oral segment of the ETT. Bacterial culture was performed via the roll-plate technique on the ETT external surface, employing blood agar (BA) and MacConkey agar. BA media was cultivated in an incubator at 35–37 °C with 5% CO2. The inoculated MacConkey media was incubated at 35–37 °C in an aerobic environment. The plates were analyzed using an automated process, with findings observed after 18 to 24 hours. Infection in the ETT was characterized by microbial growth of ≥15 colony-forming units (CFU) per semi-quantitative agar plate.

Biofilm bacterial specimens consisted of 150 µl of tryptic soy broth (TSB) media, 50 µl of 1% glucose, and 50 µl of bacterial suspension, which were placed into the microplate well. The microplate was thereafter covered with a lid and incubated for 24 hours at 37 °C. The residual solution in the incubated microplate was removed, followed by washing the microplate with a micropipette containing 150 µl of phosphate-buffered saline (PBS) solution, repeated three times. The microplate was delicately tapped to eliminate the residual solution in the well, and subsequently dried in an inverted orientation. The bacterial biofilm adhered to the well was treated with 150 µl of 95% methanol for 15 minutes, thereafter tapped lightly to remove excess liquid, and allowed to dry overnight in an inverted position at room temperature. The desiccated microplate was treated with 150 µl of a 0.1% crystal violet solution and allowed to incubate for 30 minutes. Washing with running water continued until the purple hue was no longer discernible, followed by drying the plate by inverting it at ambient temperature. Subsequent to drying, 150 µl of 70% ethanol was introduced to each well, which was then sealed and allowed to incubate for 30 minutes without shaking. We identified the biofilm by assessing its intensity, measured with optical density (OD), utilizing an ELISA microplate reader (Biorad®) at a wavelength of 495 nm. The mean OD values were computed for all examined strains and negative controls, with each being conducted in triplicate. The cut-off value (ODc) is calculated as the average OD of the negative control plus three times the standard deviation (SD) of the negative control. Biofilm classifications were established as follows: 1. Non-biofilm producer, $OD<ODc$; 2. Weak biofilm producer, $OD<OD\leq 2\times ODc$; 3. Moderate biofilm producer, $OD<OD\leq 4\times ODc$; 4. Strong biofilm producer, $4\times ODc<OD$ (**Figure 1**).

Bacterial profile statistics are presented quantitatively in tabular format. The analytical examination of biofilm formation and the length of ventilator utilization was quantified using Fisher's exact test.

Prior to initiating the study, we obtained authorization from the Research Ethics Committee of the

Faculty of Medicine at Universitas Airlangga and Dr. Soetomo General Hospital; the ethical clearance number was 1080/KEPK/VIII/2024. The confidentiality of research subject data was preserved by documenting identities solely through the respondents' initials. This data was utilized solely for scientific research reasons.

Results and discussion

Bacteria utilize biofilm development as a component of their adaptation and survival strategies, consisting of sessile polymicrobial communities encased inside a self-generated extracellular polymeric matrix, that attach to both biotic and abiotic surfaces. The matrix-encased structure supplies nutrition and oxygen, enabling bacteria to endure and thrive even in the most nutrient-deficient situations. (8) Given the protective armour and adhesive qualities conferred by biofilm development in bacteria, it might not be surprising that a wide array of dry healthcare surfaces and indwelling medical equipment in the ICU have been demonstrated to contain biofilms.

This study revealed a significant prevalence of biofilm-forming bacteria from the oral side of the external surface of ETTs, with 85% (57 out of 67) of the isolates being biofilm producers. A total of nine distinct bacterial species from 41 ETTs, comprising 67 bacterial isolates.

The Gram-negative organisms constituted a significant portion of the isolates and were predominantly biofilm producers. Klebsiella pneumoniae was the most dominant species, accounting for 33% (22/67) of isolates, with 90.9% (20/22) of them producing biofilm. Of these, a significant proportion formed strong (7/20) and moderate (7/20) biofilms. Similarly, Pseudomonas aeruginosa (14 isolates, 20.9%) displayed 92.9% biofilm positivity, with half classified as strong producers. Acinetobacter baumannii also showed high biofilm-forming capability (85.7%, or 6/7), with a tendency toward moderate (2/6) and weak (4/6) biofilm production. Although Enterobacter spp. had a lower biofilm rate (66.7%, or 4/6), most of its biofilms were strong or moderate in character. In contrast, Gram-positive bacteria were less prevalent but equally significant. Coagulase-negative Staphylococcus (CoNS) accounted for 9% (6/67) of isolates, all of which demonstrated biofilm formation. Two-thirds of the CoNS isolates (5/6) produced moderate to strong biofilms, highlighting their capacity to persist on abiotic surfaces (**Table 1**).

Biofilm formation among Gram-negative pathogens—especially K. pneumoniae, P. aeruginosa, and A. baumannii—is driven by complex virulence mechanisms including type IV pili, flagella, quorum sensing (QS), and the secretion of exopolysaccharides, proteases, and toxins. These traits facilitate surface attachment, immune evasion, and persistent colonization on ETTs. Notably, P. aeruginosa expresses elastases (LasA, LasB), pyocyanin, and rhl quorum-sensing systems (QS-regulated protease IV) to coordinate biofilm maturation and secretion of toxins, which disrupt phagocytic activity, degrade immunoglobulins, and lead to massive inflammatory damage. (9) K. pneumoniae showed robust biofilm presence in our study. This species uses type 1 and type 3 fimbriae, as well as a capsular polysaccharide, to enhance adhesion and protect against immune attack. (10) Meanwhile, A. baumannii, known for utilizing outer membrane proteins and the biofilm-associated protein (Bap), persists on device surfaces. (11)

Among Gram-positive bacteria, CoNS showed 100% biofilm production, with most forming moderate or strong biofilms. The biofilm matrix in Staphylococci is heavily influenced by PIA and MSCRAMMs, facilitating firm adhesion to polymeric surfaces like ETTs to establish resilient biofilm communities and resisting phagocytosis and complement-mediated killing. (9)

The three most prevalent bacteria identified in this study were Klebsiella pneumoniae, Pseudomonas aeruginosa, and Acinetobacter baumannii. These findings were quite surprising as they resembled the three predominant bacteria identified in the sputum culture results of all patients in the ICU of Dr. Soetomo General Hospital in 2023. (12) Although the clinically significant aspects have not been thoroughly elucidated in this study, it is imperative to enhance awareness regarding the formation of biofilms on the external surface of ETT, as dormant bacteria within these biofilms may adapt, persist, and augment their pathogenicity. Over time, mature planktonic and active microorganisms will be released from the biofilm layer, serving as the primary source of pathogens in the adjacent respiratory tract via the micro-aspiration process. (13) These planktonic microorganisms were believed to exhibit enhanced resistance and tolerance to antimicrobial treatment. (1,2)

The substantial prevalence of colonizing and biofilm-forming bacteria identified in this study may be attributed to the inadequacy of oral hygiene practices employing the currently utilized solution, chlorhexidine. The widespread and often unregulated use of chlorhexidine in healthcare settings has led to growing concerns about the emergence of bacterial resistance. Evidence from both clinical and laboratory studies indicates that repeated exposure

to sublethal concentrations of chlorhexidine can induce resistance in various pathogens, including Pseudomonas aeruginosa, Klebsiella pneumoniae, and Staphylococcus aureus, primarily through mechanisms such as efflux pump expression and membrane modifications. The alterations in the sensitivity of the bacterial cell membrane due to repeated exposure to chlorhexidine are reflected by the elevation of minimum inhibitory concentration (MIC) in cultured bacterial strains. (5) Wand et al. documented the in vitro adaptation of Klebsiella pneumoniae isolates to chlorhexidine, resulting in resistance to this agent and cross-resistance to the last-line antibiotic, colistin. (14)

The majority of ETT samples in this study were obtained from patients on a ventilator for a duration of 3 to 5 days, comprising 35 ETTs (85.4%), whereas 6 ETTs (14.6%) were collected from patients on a ventilator for 6 days or longer. The variation in the duration of ETT usage does not statistically indicate a significant difference in the presence and intensity of bacterial biofilm ($p>0.05$) (**Table 2**). Likewise, the quantity of biofilm-forming bacteria in each ETT was determined to be statistically insignificant with its utilization (**Table 3**). This aligns with prior research indicating no variation in the incidence of biofilm formation relative to the duration of medical device usage, asserting that biofilms would form even after the initial 24 hours of device application. (15,16)

ETT biofilms are frequently polymicrobial, with oropharyngeal and intestinal normal flora being present. Although oral commensals have long been thought to be non-pathogenic, experimental studies of polymicrobial infections have called this into question. It has been observed that interactions between oral commensals and Pseudomonas aeruginosa can result in enhanced virulence in respiratory infections. (2) Furthermore, coaggregation and cooperative contacts across bacterial species may encourage the production of more strong biofilms, increasing these bacteria's antibiotic resistance.

Critically ill patients exhibit a range of host immune impairments that create an environment permissive to biofilm formation: Immuno-paralysis and neutrophil dysfunction limit early pathogen clearance; (17–19) disruption of mucociliary clearance and epithelial barrier integrity due to intubation provide surfaces for microbial adhesion; (20) prolonged exposure to broad-spectrum antibiotics promotes the emergence of resistant and biofilm-forming strains. (21,22) These immunological impairments promote bacterial adhesion and biofilm maturation on the external surface of the ETT, which is frequently exposed to oropharyngeal secretions and environmen tal contaminants.

The observed high biofilm-forming ability may reflect evolved bacterial virulence mechanisms that contribute to antibiotic and antiseptic resistance. These mechanisms encompass surface adhesins and fimbriae that mediate attachment to abiotic surfaces; (23,24) quorum sensing systems that regulate virulence gene expression, biofilm density, and detachment; (25) exopolysaccharide (EPS) matrix production, which forms a barrier against immune effectors and antimicrobials (26); efflux pumps and outer membrane modifications, contributing to biofilm-associated antiseptic and antibiotic tolerance. (5,27) These findings underscore the urgent need for an evidence-based antiseptic stewardship framework aimed at optimizing chlorhexidine use, monitoring resistance patterns, and evaluating the rotation or substitution of antiseptic agents to mitigate selective pressure and preserve clinical effectiveness. (5)

The difficulties in eliminating biofilm-associated medical devices are also related to the compact structure of each biofilm, which obstructs the penetration of toxic substances, such as antimicrobials and disinfectants, preventing them from reaching the microbial cells. (28) The failure to eradicate these biofilms has emerged as a significant contributor to the incidence of infections and diseases associated with contamination issues. The heightened resistance of biofilms to disinfectants, such as chlorhexidine, has impeded their eradication in hospital settings. As recent VAP guidelines have recommended performing oral hygiene care without chlorhexidine, (29) national regulations should also provide a definitive recommendation for the substitution of this solution. Further research is required to identify alternative agents for oral hygiene that can prevent or eliminate colonization and biofilms in the oropharyngeal cavity, particularly on the surfaces of medical devices used.

Conclusion

This study demonstrated that despite the application of oral hygiene protocols using 0.2% chlorhexidine, colonization by biofilm-forming bacteria on the external surfaces of ETTs remains highly prevalent in critically ill patients. The intensity of biofilm formation did not correlate significantly with the duration of intubation, suggesting that biofilm development can occur early and is likely influenced by microbial virulence factors and host immune status. These findings underscore the limitations of chlorhexidine as a sole preventive agent and highlight the need for comprehensive strategies to mitigate biofilm-related risks in mechanically ventilated patients.

Table 1. Bacterial and biofilm profile and intensity on endotracheal tubes

Organism	Total of isolate, n (%)	Total of biofilm, n (%)	Biofilm			Non biofilm, n (%)
			Strong	Moderate	Weak	
Klebsiella pneumoniae	22 (33)	20 (90.9)	7	7	6	2 (9.1)
Pseudomonas aeruginosa	14 (20)	13 (92.9)	6	6	1	1 (7.1)
Acinetobacter baumannii	7 (10)	6 (85.7)	0	2	4	1 (14.3)
Coagulase negative Staphylococcus sp.	6 (9)	6 (100)	2	3	1	0
Enterobacter	6 (9)	4 (66.7)	3	1	0	2 (33.3)
Streptococcus viridans	4 (6)	2 (50)	0	2	0	2 (50)
Escherichia coli	5 (7,5)	3 (60)	2	1	0	2 (40)
Staphylococcus aureus	2 (3)	2 (100)	0	2	0	0
Proteus	1 (1.5)	1 (100)	0	0	1	0
Total, n (%)	67 (100)	57 (85)	20 (35)	24 (42)	13 (23)	10 (15)

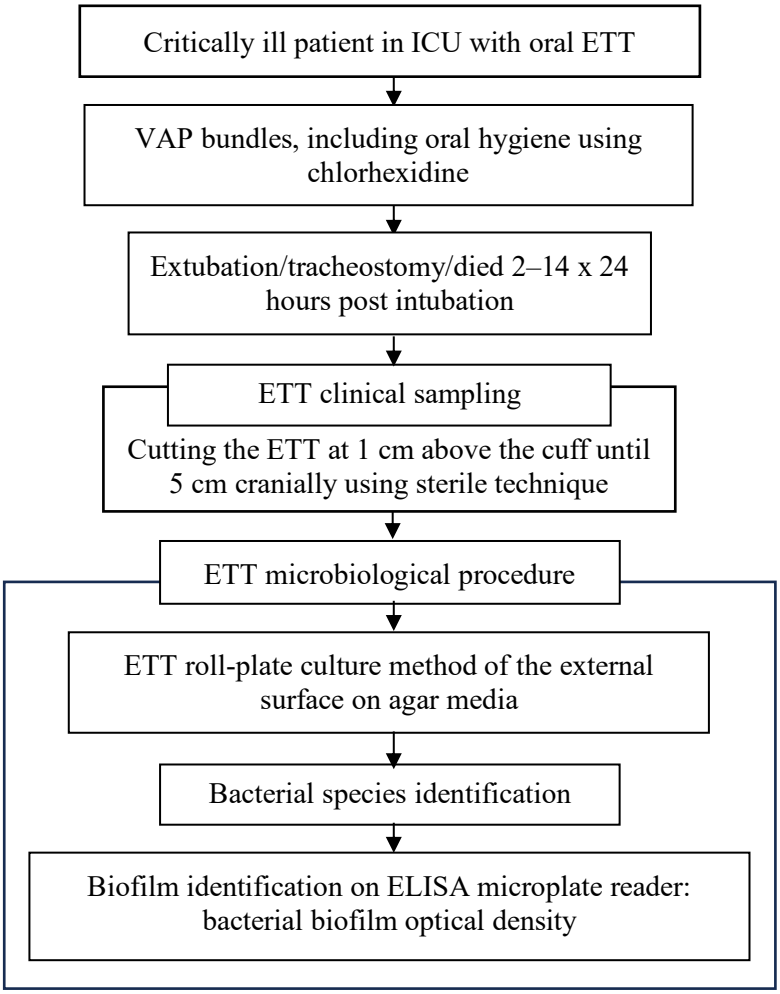
Table 2. The correlation between the length of treatment and biofilm formation

Biofilm type	Detected bacterial biofilms		p value
	<6 days	≥6 days	
Non-biofilm, n (%)	10 (17.2)	-	0.663
Weak, n (%)	11 (18.9)	2 (22.2)	
Moderate, n (%)	20 (34.5)	4 (44.4)	
Strong, n (%)	17 (29.3)	3 (33.3)	
Total, n	58	9 (13.4)	

Table 3. Correlation between the length of treatment and the number of bacteria forming biofilm

Biofilm type	The number of bacterial biofilms detected		p value
	<6 days	≥6 days	
Monomicrobial, n (%)	14 (40)	3 (50)	0.679
Polymicrobial, n (%)	21 (60)	3 (50)	
Total, n	35	6	

Figure 1. Schematic diagram of research design



Legend: ICU=intensive care unit; ETT=endotracheal tube; VAP=ventilator-associated pneumonia; ELISA=enzyme-linked immunosorbent assay.

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