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Implications of endotracheal tube biofilm for ventilator-associated pneumonia

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Abstract *Objective*: To determine the relationship between, and antibiotic resistance of, endotracheal tube (ET) biofilm and pulmonary pathogens in ventilator-associated pneumonia (VAP).
Setting: General intensive care units in two university teaching hospitals.
Design: The microbiology of ET biofilm and tracheal samples from patients with and without VAP were compared. For individual patients, matching pairs of pathogens were confirmed as identical and characterised for antibiotic susceptibility.
Patients: 40 intensive care unit patients – 20 with VAP, 20 without VAP as control. The duration of intubation (median and range) was 6.5 days (3–17) and 5 days (2–10), respectively.
Measurements and results: Samples of tracheal secretions were taken during ventilation for bacteriological culture. Following extubation, ETs were examined for the presence of biofilm. Isolates of high pathogenic potential included *Staphylococcus aureus*, enterococci, *Enterobacteriaceae*, pseudomonads and *Candida* spp. Where the same microorganism was found on tracheal and ET samples by phenotyping, these were confirmed as identical by genotyping and characterised for antibiotic susceptibility in both the free floating and biofilm forms. Seventy per cent of patients with VAP had identical pathogens isolated from both ET biofilm and tracheal secretions. No pairing of pathogens was observed in control patients ($p < 0.005$). Susceptibility data for these pairs show that the ET acts as a reservoir for infecting microorganisms which exhibit significantly greater antibiotic resistance than their tracheal counterparts.
Conclusion: This investigation provides further evidence for the role of ET biofilm in VAP. The difficulty in eradicating an established microbial biofilm using antibiotics implies that increased attention must be directed towards modification of the ET to prevent or substantially reduce biofilm formation.

Key words Ventilator-associated pneumonia · Microbial biofilm · Antibiotic resistance · Endotracheal tube · Medical device

Introduction

The endotracheal tube has been cited as a possible reservoir for infecting microorganisms in the respiratory tract [1–5]. As an implanted foreign body, the endotracheal tube (ET) is acknowledged to constitute a risk factor for ventilator-associated pneumonia (VAP) by bypassing host defences and allowing inhaled particles direct access to the airways. It has been shown that microorganisms can adhere to the surface of the ET and some species exude an exopolysaccharide which acts as a slime-like adhesive. Bacteria encased in this matrix

(biofilm) are relatively resistant to the action of antimicrobials and host defences [4, 6]. This situation arises partly because microorganisms within the biofilm are in a nutrient-depleted state and therefore metabolically quiescent. This renders them relatively insensitive to the effects of antibiotics. In addition, several studies have shown that the exopolysaccharide can prevent penetration of antibiotics into the biofilm [6, 7]. It has been suggested that dissemination of such microorganisms from the biofilm into the airways may occur upon insertion of a suction catheter. Fragments of ET biofilm may be dislodged and carried further into the lung by ventilator gas flow [8].

No direct evidence has been published linking the ET and microbial biofilm with VAP in the ICU setting. Therefore, the aim of this study is to determine the importance of ET biofilm in VAP and to characterise the susceptibility of biofilm isolates to antimicrobials routinely used as prophylaxis or empirical therapy.

Patients and methods

Forty patients undergoing mechanical ventilation in the intensive care unit (ICU) at the Royal Victoria Hospital, Belfast and Belfast City Hospital were recruited to the study: 20 patients with VAP and 20 without VAP. All patients were from medical or surgical services, were assessed using the Acute Physiology and Chronic Health Evaluation (APACHE II) scoring on admission and received antibiotics as prophylaxis or treatment during their stay in the ICU. In addition, patients were given routine prophylaxis for stress ulceration in the form of H_2 receptor antagonists with antacid as required.

Patients were excluded from the study if they were discharged from the ICU or developed pneumonia within 48 h of admission, were readmitted to the ICU, had been transferred from the coronary care or burns unit or suffered multiple trauma. Patients were diagnosed as having VAP if body temperature was greater than 37.5 °C, white cell count was greater than 12×10^9/l or less than 4×10^9/l and new or progressive infiltrates were seen on chest radiograph. In addition, patients were required to have either new onset of purulent sputum or a significant pathogen isolated from pulmonary secretions [9]. Infections beginning less than 48 h after intubation were defined as nosocomial infection rather than VAP and these patients were excluded from investigation.

Microorganisms considered to be of high pathogenic potential included *Staphylococcus aureus*, *Enterobacteriaceae*, pseudomonads and yeast. *Enterococcus* colonisation was also recorded. Microorganisms such as coagulase-negative staphylococci, viridans streptococci and diphtheroid bacilli were not considered to be of high pathogenic potential and therefore not included in the data analysis.

Endotracheal tubes

All ETs were made of polyvinyl chloride (PVC) and of the low-pressure high-volume cuffed type. Following extubation, ETs were immediately placed in a sealed specimen bag, stored at 4 °C and processed within 6 h of extubation. Two 1-cm sections were cut from the tip region, just above the cuff and from the distal end.

Isolates were removed from the ET biofilm by a sequence of scraping, vortex-mixing for 1 min in 15 ml of sterile 1/4 strength Ringer's solution and gentle ultrasonication (3×10 s pulses, 150 W ultrasonic bath nominal frequency 50 Hz). These procedures are not detrimental to the entrained microorganisms [10]. Suspensions derived from ET biofilm were streaked onto horse blood, chocolate blood and MacConkey agars (Oxoid, Basingstokes, Hants, UK). Agar plates were examined for single colony formation after incubation for 48 h at 37 °C in a 10 % carbon dioxide-enriched atmosphere.

Tracheal secretions

Tracheal secretions were collected every 2 days during the period of intubation by aspiration into a sputum trap. The aspirate was digested to form a homogeneous suspension using an equal volume of Sputasol (Oxoid, Basingstoke, Hants, UK). Using sterile saline, 1/100 and 1/10 000 dilutions of the homogenised suspension were prepared and 100 µl of each dilution was cultured onto appropriate media. Only the 1/100 dilution was plated onto MacConkey agar to facilitate recognition of *Enterobacteriaceae*. Cultures were incubated at 37 °C in an atmosphere of room air with 10 % carbon dioxide enrichment and examined after 24 and 48 h [11]. A threshold of 10^5 colony-forming units (CFU)/ml was used to distinguish infection from colonisation. Only pathogens present in numbers greater than 10^5 CFU/ml were used in subsequent analyses. Pathogens reported are those detected on the day on which VAP was first diagnosed.

Identification and subspecies characterisation of isolates

Isolates were compared initially employing appropriate phenotyping techniques [11–13]. When the same microorganism was detected on the ET and tracheal samples by phenotyping, further testing was undertaken to determine if these isolates were identical at the genome level. Only isolates which were identical at the genome level were considered matching pairs. Genotyping employed pulsed-field gel electrophoresis (PFGE) and arbitrary-primed polymerase chain reaction (AP-PCR) or random amplification of polymorphic DNA technique. For PFGE analysis, bacterial DNA was extracted and digested with the restriction enzyme Sma1 [14]. Resultant fragments were compared using contour-clamped homogeneous electric field electrophoresis [15]. PCR was used to amplify anonymous regions of chromosomal DNA using a 25-mer random primer as described previously [16]. Resulting banding patterns 300–2000 bp were visualised by 2 % agarose electrophoresis under ultraviolet light and stained with ethidium bromide. These data were confirmed by employing four additional arbitrary oligonucleotide DNA primers.

Susceptibility testing

Susceptibility testing was performed on the matching pairs of tracheal and ET isolates. The usual laboratory practice for determining microbial susceptibility to antibiotics by reporting minimum inhibitory concentrations (MIC) does not adequately describe in vivo susceptibility for biofilm [17]. MICs are determined on microorganisms which exist as individual cells, suspended in growth medium. These cells are said to be in planktonic growth mode and tend to show greater susceptibility to the action of antimicrobial agents. However, when present as a biofilm on the ET, pathogens exist in a sessile growth mode and are more resistant to the action

of antimicrobials for reasons which include their metabolic quiescence and protection from host defences and biocides by the encasing exopolysaccharide. Therefore, to measure susceptibility accurately, in vivo, microorganisms should be tested in this sessile form, within an established biofilm; the methodology permitting only minimum bactericidal concentrations (MBC) to be determined.

Antibiotics commonly administered as prophylaxis or treatment regimens in our ICUs (tobramycin, cefotaxime and cefuroxime) were used in susceptibility testing. MICs were determined for ET and tracheal isolates in the suspended, or planktonic form as a measure of conventionally accepted susceptibility [17]. MBCs for the tracheal isolates, also in planktonic growth mode, were determined for tobramycin, cefotaxime and cefuroxime by standard methodology [17]. In contrast, MBCs were determined for these antibiotics against ET microorganisms in the biofilm mode of growth. Biofilm was formed by incubating pathogen monocultures (10^7 CFU/ml) with 1-cm^2 discs of medical grade PVC in MHB for 24 h at 37 °C. Biofilm formation on discs was confirmed by scanning electron microscopy. Discs were then removed, washed with phosphate buffered saline (PBS) and incubated in PBS with antibiotic (24 h at 37 °C). Discs were again removed and washed with PBS. Adherent microorganisms were removed from the PVC discs as previously described and MBCs determined [17].

Statistical analysis

Statistical evaluation of the association between pairs of isolates derived from biofilm and tracheal secretions in control and pneumonia groups was performed using a 2×2 experimental design in conjunction with Fisher's exact probability test. Patient data were compared using the Mann-Whitney U test. Comparisons of susceptibility data (MICs and MBCs) between matching tracheal and ET isolates were evaluated using the Wilcoxon signed rank test. For the purpose of this study, $p < 0.05$ was accepted to denote significance [18].

Results

Details of the patients studied are provided in Table 1. There were no significant differences between control and VAP groups for age ($p = 0.93$), duration of intubation ($p = 0.45$) and severity scoring ($p = 0.68$)

Microbial biofilm was isolated from all ETs removed from both control patients and those with VAP. However, microorganisms of high pathogenic potential were isolated from all ETs collected from patients with VAP compared with 30 % of ETs from the control group. In tracheal secretions, microorganisms of high pathogenic potential were recovered from 85 % of patients in the group with VAP and from 20 % of patients serving as controls. Further details of these pathogens are presented in Table 2.

Of those patients with VAP, 70 % had pathogens which were indistinguishable by genotyping in both ET biofilm and tracheal secretions. In contrast, no matching pairs of pathogens were isolated from control patients. Data analysis (Fisher's exact probability test) indicated that there was a highly significant ($p < 0.005$) association between the pairs of microorganisms isolated from patients with VAP. These matching pairs of isolates were *Pseudomonas aeruginosa* (5), *Staphylococcus aureus* (4), EGNB (3: *Proteus mirabilis*, *Klebsiella pneumoniae* and *Enterobacter*), *Enterococcus faecalis* (1) and *Candida* spp. (1).

MICs and MBCs of tobramycin, cefotaxime and cefuroxime, determined for the matching ET (13 bacterial isolates) and tracheal isolates, are given in Table 3. Susceptibility data for *E. faecalis* has not been included in Table 3 as, in all cases, MICs and MBCs were in excess of 1024 mg/l. Data analysis showed that MBCs determined for tracheal isolates were significantly lower than those from isolates in biofilm growth mode for tobramycin ($p = 0.002$), cefotaxime ($p = 0.02$) and cefuroxime ($p = 0.02$). In contrast, when susceptibility testing was undertaken in the planktonic form, there were no significant differences between MICs from ET- and tracheal-derived isolates.

Table 1 Patient details

	VAP	Control
Number (male/female)	20 (11/9)	20 (10/10)
Patient age (years) (median and range)	62 (17–79)	53 (17–82)
Duration of intubation (days) (median and range)	6.5 (3–17)	5 (2–10)
APACHE II score (median and range)	19 (8–26)	17 (2–21)
Number of patients from:		
Medical	4	6
Postsurgery	16	14

Table 2 Numbers of isolates of potential pathogens recovered from endotracheal tube biofilm and tracheal secretions

	Control		VAP	
	Biofilm	Tracheal	Biofilm	Tracheal
Pseudomonas aeruginosa	–	–	6	6
EGNB	2	3	4	5
Enterococcus faecalis	2	–	2	2
Staphylococcus aureus	2	–	6	6
Candida spp.	1	2	4	2

Discussion

The presence of ET biofilm in all patients undergoing ventilation in the ICU indicates how readily microorganisms can adhere to biomaterials. The clinical significance of this observation in the aetiology of VAP has

Table 3 Susceptibility data for matching pairs[a] of tracheal and ET isolates

Microorganism	Tobramycin (mg/l)		Cefotaxime (mg/l)		Cefuroxime (mg/l)	
	MIC	MBC[b]	MIC	MBC[b]	MIC	MBC[b]
P. aeruginosa						
Tracheal	2 (2–32)	256 (64–512)	16 (32–128)	32 (32–128)	256	> 1024
ET	4 (4–64)	**> 1024**	128 (32–256)	**> 1024**	512	**> 1024**
Enterobacteriaceae						
Tracheal	4 (2–8)	4 (4–16)	4 (< 0.5–16)	128 (32–128)	16 (4–32)	32 (32–128)
ET	2 (1–4)	**> 1024**	32 (0.5–64)	**256 (32–> 1024)**	16 (8–256)	**512 (256–> 1024)**
S. aureus						
Tracheal	1 (1–8)	18 (2–512)	4 (2–4)	16 (16–256)	< 0.5 (< 0.5–64)	16 (16–256)
ET	3 (1–8)	**> 1024 (256–> 1024)**	2 (2–4)	**128 (64–> 1024)**	1.5 (1–64)	**> 1024 (512–> 1024)**

[a] 13 matching bacterial isolates from 13 patients were used for susceptibility testing

[b] MBCs shown in bold were obtained using ET isolates in biofilm growth mode. The other MBCs were obtained from the corresponding tracheal isolates in suspended (planktonic) growth mode

been unclear [1–5, 19]. This study provides further evidence implicating the ET as a reservoir for infecting pathogens which are insensitive to the action of antimicrobial agents when in the sessile form. We have shown that the ET from patients with VAP is more frequently contaminated by pathogens than ETs from control patients. Indeed, 70% of patients with VAP had the identical pathogen isolated from the ET and lower respiratory tract, although no matching pairs of isolates were found in the control group.

It is difficult to determine definitively whether ET colonisation precedes that of the airways. It would not be ethical to extubate patients each day in order to examine the ET for biofilm. Nor is it possible to remove biofilm with the ET in situ using, for example, a luminal brush. Indeed, this would only serve to contaminate sampling by mucus and secretions from the airways. We were, therefore, obliged to examine the ET after extubation. However, our previous work has shown that ET biofilm develops within hours of intubation, with pathogens present in high concentrations [3]. It is, therefore, probable that early ET colonisation, with large numbers of microorganisms resistant to the actions of antibiotics, precedes colonisation of the airways with planktonic microorganisms sensitive to antimicrobials. Inglis [8] has demonstrated how aggregates of ET biofilm can become detached from the main body of biofilm by suction catheter and disseminate towards the lower respiratory tract. Such aggregates have been shown to be considerably more pathogenic than individual aerosolised cells in producing VAP in animal models [20, 21]. That a dense and often polymicrobial flora was universally present on ETs would support the view that the ET may act as a reservoir for pathogens.

The presence of pathogens on the ET may occur as a result of gastropulmonary reflux, or from a colonised oropharynx [22–24]. Several factors favour ET colonisation in the critically ill patient. For example, small numbers of microorganisms can adhere to surfaces, such as the ET, and rapidly secrete a glycocalyx, offering protection from antimicrobials and host defences [3,4]. This process begins within 6 h of the patient being intubated and the protected environment of the biofilm permits proliferation of pathogens. Indeed, the subinhibitory concentrations of antibiotics often detected in the airways [4, 25, 26] may facilitate the initial adherence of pathogens to the ET [27]. Once established, microorganisms within the ET biofilm are difficult to eradicate.

The sessile growth mode observed in biofilm has important implications for antimicrobial testing and selection. While clinical decisions are often based on MICs using microorganisms in suspension in vivo, they exist in a different growth mode which markedly alters their susceptibility pattern, rendering them less susceptible to the action of antibiotics (Table 3). Clearly, there exists a need to develop methods which more accurately can assess microbial susceptibility within the clinical laboratory [6].

This study provides further evidence to implicate the ET biofilm in VAP. The biofilm adherent to the ET provides a protected environment for potentially pathogenic microorganisms which may be transported into the pulmonary tree and give rise to infection. Attempts at preventing the attachment of pathogens to the ET by incorporating antibiotics into biomaterials has met with little success. However, other methods such as biomaterial surface modification and deposition of antiadherent coatings may provide alternative, more productive routes to reducing the adherent potential of microorganisms to the ET [28–30].

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