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Eradication of endotracheal tube biofilm by nebulised gentamicin

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Abstract *Objective*: To compare the efficacy of gentamicin, nebulised via the endotracheal tube (ET), with that of parenteral cefotaxime or parenteral cefuroxime in preventing the formation of ET biofilm. *Setting*: General intensive care units in two university teaching hospitals. *Design*: The microbiology of ET biofilm from 36 ICU patients eligible to receive antibiotic prophylaxis was examined. Peak and trough tracheal concentrations of gentamicin, cefotaxime or cefuroxime were measured in each patient group, on the 2nd day of intubation. *Patients*: Twelve patients received gentamicin (80 mg) nebulised in 4 ml normal saline every 8 h, 12 cefotaxime (1 g, 12 hourly) and 12 cefuroxime (750 mg, 8 hourly). Prophylaxis was continued for the duration of intubation. *Measurements and results*: Samples of tracheal secretions were taken on the 2nd day of ventilation for determination of antibiotic concentrations. Following extubation, ETs were examined for the presence of biofilm. Pathogens considered to be common aetiological agents for VAP included *Staphylococcus aureus*, enterococci, Enterobacteriaceae and pseudomonads. While microbial biofilm was found on all ETs from the cephalosporin group, microbial biofilm of these micro-organisms was found on 7 of the 12 ET tubes from patients receiving cefotaxime [*S. aureus* (4), pseudomonads (1), Enterobacteriaceae (1), enterococcus (1)] and 8 of the 12 ET tubes from patients receiving cefuroxime [Enterobacteriaceae (6), *P. aeruginosa* (1) and enterococcus (1)]. While microbial biofilm was observed on five ETs from patients receiving nebulised gentamicin, none of these were from pathogens for ventilator-associated pneumonia (VAP). Tracheal concentrations of both cephalosporins were lower than those needed to inhibit the growth of pathogens recovered from ET tube biofilm. The median (and range) concentrations for cefotaxime were 0.90 (<0.23–1.31) mg/l and 0.28 (<0.23–0.58) mg/l for 2 h post-dose and trough samples, respectively. Two hours post-dose concentrations of cefuroxime (median and range) were 0.40 (0.34–0.83) mg/l, with trough concentrations of 0.35 (<0.22–0.47) mg/l. Tracheal concentrations (median and range) of gentamicin measured 1 h post-nebulisation were 790 (352–>1250) mg/l and then, before the next dose, were 436 (250–1000) mg/l. *Conclusion*: Nebulised gentamicin attained high concentrations in the ET lumen and was more effective in preventing the formation of biofilm than either parenterally administered cephalosporin and therefore may be effective in preventing this complication of mechanical ventilation.

Keywords Ventilator-associated pneumonia · Microbial biofilm · Endotracheal tube · Medical device · Nebulised gentamicin

Introduction

Microbial growth in biofilm form poses a difficult problem to overcome. Within the context of intubated intensive care patients, microbial biofilm is most frequently encountered on the endotracheal tube (ET). Thus, intubation constitutes a factor risk for ventilator-associated pneumonia (VAP) in two ways. Firstly, as an implanted foreign body, the ET by-passes host defences and allows inhaled micro-organisms direct access to the airways [1, 2]. Secondly, microbial biofilm on the ET surface provides a reservoir of infecting micro-organisms [3, 4] which are greatly resistant to the action of antibiotics because of their metabolic quiescence and the protection from host defences and biocides afforded by the encasing exopolysaccharide [5, 6, 7]. To date, antibiotic therapy has not been successful in preventing biofilm formation or in eradicating an established biofilm [8, 9] on the ET. The use of non-antibiotic approaches, such as modification of the ET by deposition of anti-adherent coatings on its surface, has yet to deliver the promised benefits [7].

While antibiotic prophylaxis for VAP remains a controversial area, we recently assessed nebulised gentamicin for use as prophylaxis for VAP in high-risk intubated patients [10]. This antibiotic was shown to undergo minimal systemic absorption from the airways following delivery by this route, did not accumulate over time and was well tolerated by patients. In addition, nebulised gentamicin can bring about significant improvement in clinical and airway inflammatory indicia in mechanically ventilated patients [11]. Given that nebulised antibiotic is likely to achieve higher pulmonary concentrations than an antibiotic administered by the parenteral route, the aim of this investigation was to compare the efficacy of conventional antibiotic prophylaxis for VAP (parenteral cefotaxime or cefuroxime) with that of nebulised gentamicin in preventing the formation of microbial biofilm on the ET.

Patients and methods

Thirty-six mechanically ventilated patients in the intensive care unit (ICU) at the Belfast City Hospital and Royal Victoria Hospital were studied. All of the patients, from medical or surgical services, were assessed using APACHE II scoring on admission. Patients eligible to receive antibiotic prophylaxis were those in whom ventilation was expected to last more than 48 h. Indications for antibiotic prophylaxis were immunocompromise (neutropenia), leucopenia and the administration of high-dose corticosteroids. The study did not use a randomised design as 12 patients received nebulised gentamicin (used in the Belfast City Hospital), 12 patients cefotaxime and 12 cefuroxime (Royal Victoria Hospital). Two patients receiving gentamicin also received metronidazole and, in the cephalosporin group, three patients received metronidazole, one benzylpenicillin, one flucloxacillin and one ciprofloxacin.

Gentamicin (80 mg) was nebulised in 4 ml normal saline, by nebuliser, every 8 h as antibiotic prophylaxis during ventilation. A Bennett 7200 series ventilator with standard nebulising system was used within a closed system fitted with filters on the expiratory limb of the ventilator to prevent re-entry of gentamicin into the ventilator. The nebulisation time was approximately 20 min and the particle size generated was less than 10 µm. The dose of 80 mg was given regardless of the patient's age and weight. The dose of cefotaxime was 1 g, 12 hourly, and for cefuroxime 750 mg, 8 hourly. Prophylaxis was continued for the duration of intubation and all patients were given routine prophylaxis for stress-ulceration in the form of H_2-receptor antagonists.

Collection of tracheal aspirate

For determination of antibiotic concentrations, samples of tracheal aspirate were taken via a sputum trap after patients has been receiving antibiotic for at least 24 h. Samples were taken immediately before the morning dose (trough sample) and 2 h after the dose of cephalosporin was administered (post distribution phase) or 1 h after gentamicin had been nebulised. Following collection, samples were stored at –70°C prior to their analysis for antibiotic.

Endotracheal tubes

All ETs were polyvinyl chloride 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. Secretions and mucus were washed from the ET using sterile phosphate-buffered saline after which two 1 cm sections were cut from the tip region, just above the cuff and from the distal end. Each of these were split longitudinally, with one section removed for microbial identification and the other used for visual confirmation of biofilm by electron microscopy. This method of processing ETs ensured that any gentamicin carryover was minimal and sub-minimum inhibitory concentration (MIC).

For electron microscopy, sections of ET in glutaraldehyde fixative were processed as previously described prior to examination under a JEOL 6400 scanning electron microscope [12].

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 ultrasonic treatment (3×10 s pulses, 150 W ultrasonic bath nominal frequency 50 Hz). These procedures are not detrimental to the entrained micro-organisms [12]. Suspensions derived from ET biofilm were streaked onto horse blood, chocolate blood and MacConkey agars (Oxoid, Hants). Agar plates were examined for single colony formation after incubation for 48 h at 37°C in a 10% carbon dioxide-enriched atmosphere. If sub-culturing was required for isolation, this was kept to a minimum in order to preserve bacterial cell surface characteristics. If the secondary plate revealed impure culture it was discarded and the primary plate sub-cultured again.

Microorganisms considered aetiological for VAP included *Staphylococcus aureus*, Enterobacteriaceae, enterococci, pseudomonads and *Enterococcus*. While types of yeast may be classified as aetiological micro-organisms for VAP, the fact they are routinely present as oropharyngeal flora make it difficult to ascertain the clinical significance of positive cultures. Thus, the presence of yeast was only classified as a potential pathogen if the micro-organism was isolated from two different sites in the patient, including the respiratory tract, as part of normal clinical surveillance. Micro-organisms such as coagulase-negative *Staphylococci* (CNS), *Streptococci viridans* and diphtheroid *Bacilli* were not considered to be of high pathogenic potential for VAP and thus not recorded in the results [3].

Identification and storage of isolated micro-organisms

All gram-positive cocci were catalase-tested. Those testing positive were regarded as staphylococci and were both slide coagulase tested by the Staph aurex slide agglutination method (Wellcome, UK) and tube coagulase-tested. Organisms testing positive to both the slide and tube coagulase tests were identified as *S. aureus.* All other catalase-positive, coagulase-negative isolates were identified as CNS. All catalase-negative gram-positive cocci were tested for Lancefield carbohydrate group antigens A, B, C, D, F and G as previously described [3].

All gram-negative rods were oxidase-tested, oxidase-positive organisms being identified by the API 20 NE system and oxidase-negative micro-organisms by the API 20 E system. Gram-negative rods exhibiting swarming from morphology were sub-cultured onto urease slopes. The appearance of characteristic budding together with typical cream colonies 4–6 mm in diameter with elevated colonial morphology was considered in itself sufficient identification of fungi/yeast.

A colony of the identified isolate was inoculated into the cryopreservative fluid of a Protect Bacterial Preserve System (Technical Service Consultants, UK). These were stored at –20°C following vigorous shaking to ensure even dispersion of the organism.

Susceptibility testing

Isolates recovered from each patient group were tested for susceptibility to that antibiotic and minimum inhibitory concentrations (MICs) determined for antibiotic prophylaxis used in this study. Stock solutions of each antibiotic were prepared with sterile-distilled water and a macro-dilution method [9] employed for determination of the MIC. Doubling dilutions of the antibiotics were prepared in 5 ml volumes of Mueller-Hinton broth (Oxoid, UK) giving concentrations in the range 1024–0.01 mg/l. Isolates, grown to late exponential phase, as determined by growth curve measurements, were adjusted initially to an optical density at 540 nm of 0.4 to produce a final concentration of approximately 1×10^5 cfu/ml of each antibiotic broth dilution. After 24 h incubation at 37°C the MIC was taken to be the lowest concentration of antibiotic preventing growth of the isolate. All determinations were made in triplicate.

Analysis of gentamicin

Gentamicin was measured using fluorescence polarisation immunoassay (TDX, Abbott Laboratories, UK). Samples of aspirate were homogenised with an equal volume of Sputasol (Oxoid, Basingstoke, Hants) and 100 µl aliquots used for analysis. A calibration curve was constructed using a range of gentamicin concentrations (0, 0.5, 1.5, 3.0, 6.0 and 10 mg/l). When study samples yielded concentrations in excess of the calibration range, the sample was diluted and re-tested. Replicate measurements indicated that within-run variation was less than 6.2% and between-run variation was less than 4.8%. The correlation coefficient for the standard curve was 0.99. The limit of detection of the assay was 0.1 mg/l.

Analysis of cefotaxime and cefuroxime

Tracheal concentrations of antibiotic were determined by high-performance liquid chromatography (HPLC) [13]. The HPLC comprised a Shimadzu LC 6A pump and a Shimadzu SPD 6A UV spectrophotometric detector set at 254 nm for measurement of cefotaxime and 275 nm for cefuroxime (Shimadzu, Kyoto, Japan). Chromatograms were recorded on a Shimadzu C-R3A chromatopac computing integrator (Shimadzu, Kyoto, Japan). Separation was performed on a Nova Pak liquid chromatography cartridge C_{18} column (10 cm×8 mm (i.d.); 10 µm particle size. Waters Associates, Mass., USA). Analysis was performed on a LiChrosorb C18 column (25 cm×4.6 mm (i.d.); 10 µm particle size. HPLC Technology, Macclesfield, Cheshire, UK) which was maintained at a temperature of 28°C using a column heater (Jones Chromatography, Cardiff, UK).

The phosphate buffer was prepared by dissolving 15.6 g of sodium dihydrogen orthophosphate (BDH, Poole, UK) in 900 ml of water. A 5 M solution of sodium hydroxide solution (BDH, Poole, UK) was added in quantities sufficient to achieve pH 6.0, and the resulting solution made up to 1 l using water. The mobile phase used for cefotaxime and cefuroxime consisted of the sodium phosphate buffer (pH 6.0) containing 16% and 20% methanol, respectively (Labscan, Stillorgan, Dublin). All materials were of HPLC grade and were filtered through a 0.45 µm filter prior to use. The flow rate in both analyses was 1.7 ml/min with a retention time for cefotaxime and cefuroxime of 6.0 min and 8.9 min, respectively. One millilitre aliquots of each sample of aspirate were mixed with 40 µl of perchloric acid and the resulting mixture vortex-mixed for 30 s prior to centrifugation at 14,000 G (MSE, Loughborough, UK) for 10 min. The clear supernatant was removed and stored at –20°C prior to injection of 20 µl aliquots onto the column.

Between-day variation in the assay for antibiotics in saliva at high (5 mg/l), medium (1 mg/l) and low (0.5 mg/l) concentrations gave coefficients of variation of 1.58%, 2.62% and 2.66%, respectively, for cefotaxime and 2.20%, 14.23% and 12.26%, respectively, for cefuroxime. Both assays were linear (r=0.9998) over the range 0.5–5 mg/l. The limits of detection for the assays, based on twice peak-to-peak baseline noise were 0.22 mg/l (cefotaxime) and 0.23 mg/l (cefuroxime).

Statistical analysis

Statistical comparison of trough and post-dose antibiotic concentrations and each patient's post-dose antibiotic concentration with the corresponding MIC of isolates from ET tube biofilm was undertaken using the Wilcoxon signed rank test. For the purpose of this study, p less than 0.05 was accepted to denote significance.

Results

Clinical details of the 36 mechanically ventilated patients investigated are provided in Table 1. There were no significant differences between patient groups for age (p=0.93), duration of intubation (p=0.45) and severity scoring (p=0.68).

Endotracheal tube biofilm was observed on 5 of the 12 ETs from patients receiving gentamicin, although none of these were formed by micro-organisms considered to be aetiological for VAP; the recovered isolates consisted of CNS, Streptococcus spp. and Candida spp. Biofilm was observed, by electron microscopy, as a mass of matrix-enclosed bacterial cells which appeared to project from the confluent accretion on the luminal surface of the ET in such a manner that it could be dislodged readily and aspirated into the lower respiratory tract. In some instances, distinct rod-shaped or coccoid bacteria were observed within or on the biofilm matrix (Fig. 1).

Tracheal concentrations (median and range) of gentamicin measured immediately before the morning dose

Table 1 Patient details

	Gentamicin	Cefotaxime	Cefuroxime
Number (male/female)	12 (5/7)	12 (6/6)	12(7/5)
Patient age (years; median and range)	60 (21–84)	62 (17–79)	53 (17–82)
Duration of intubation (days; median and range)	6 (2–32)	6 (2–13)	5 (2–15)
APACHE II score (median and range)	17.5 (10–22)	19 (7–24)	15 (8–21)
Number of patients from			
Medical	7	6	9
Post surgery	5	6	3

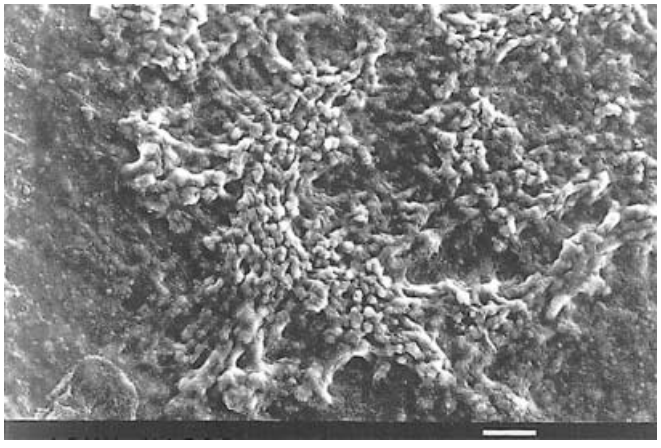

Fig. 1 A microbial biofilm from an ET showing bacteria, 1–2 μm in length (bar 10 μm)

Table 2 Tracheal concentrations of antibiotic

	Pre-dose (mg/l)[a]	Post-dose (mg/l)[a]
Gentamicin	436 (250–1000)	790 (352–>1250)
Cefotaxime	0.28 (<0.23–0.58)	0.90 (<0.23–1.31)
Cefuroxime	0.35 (<0.22–0.47)	0.40 (0.34–0.83)

[a] Median and range

Table 3 Susceptibility data for micro-organisms of clinical significance isolated from ET tube biofilm of patients receiving cephalosporins

Micro-organism	Cefotaxime		Cefuroxime	
	No. isolates	MIC (mg/l)[a]	No. isolates	MIC (mg/l)[a]
S. aureus	4	2 (2–4)	0	–
Enterobacteriaceae	1	32	6	16 (8–256)
Pseudomonads	1	16	1	32
Enterococcus	1	>1024	1	>1024

[a] median and range

and measured 1 h post-nebulisation are given in Table 2. Comparison of the pre- and post-dose data for gentamicin showed post-nebulisation concentrations to be significantly higher (p=0.0063) than the concentrations measured immediately before the next dose was administered. Individual variation for pre-and post-dose concentrations is shown in Fig. 2.

Microbial biofilm was observed on all ETs recovered from patients receiving cephalosporins. In the cefotaxime group, micro-organisms included *S. aureus* (4), *P. aeruginosa* (1), Enterobacteriaceae (1) and enterococcus (1). In contrast, colonisation of ET tubes from patients receiving cefuroxime was predominantly Gram-negative [Enterobacteriaceae (6), *P. aeruginosa* (1)], with one isolate of enterococcus. ET biofilm from remaining patients was composed of either CNS or yeast. A summary of the MICs for cefotaxime and cefuroxime for pathogens isolated from ET biofilm are shown in Table 3.

Tracheal concentrations of both cephalosporins were significantly lower than the concentrations needed to inhibit the growth of pathogens recovered from ETs, and showed wide variation. The median concentrations (and range) for cefotaxime and cefuroxime are shown in Table 2. Individual concentrations for each antibiotic are shown in Fig. 2. Concentrations of antibiotics were below the limits of detection in 5 of the 12 patients receiving cefotaxime and 2 of the 12 patients receiving cefuroxime. Statistical comparison between pre- and post-dose concentrations for each antibiotic showed significant differences for cefotaxime (p=0.0015) and cefuroxime (p=0.0055). In addition, MICs for biofilm isolates were significantly higher than the corresponding tracheal concentrations for cefotaxime (p=0.018) and cefuroxime (p=0.012).

Discussion

Previous investigations have demonstrated that ET biofilm is a risk factor for the development of VAP [3, 4]. Microbial biofilm begins to form within a few hours of patients being intubated [8, 9] and, once established, micro-organisms within the biofilm are difficult to eradicate using antibiotic therapy [5, 6]. Thus, early colonisation of the ET with large numbers of micro-organisms re-

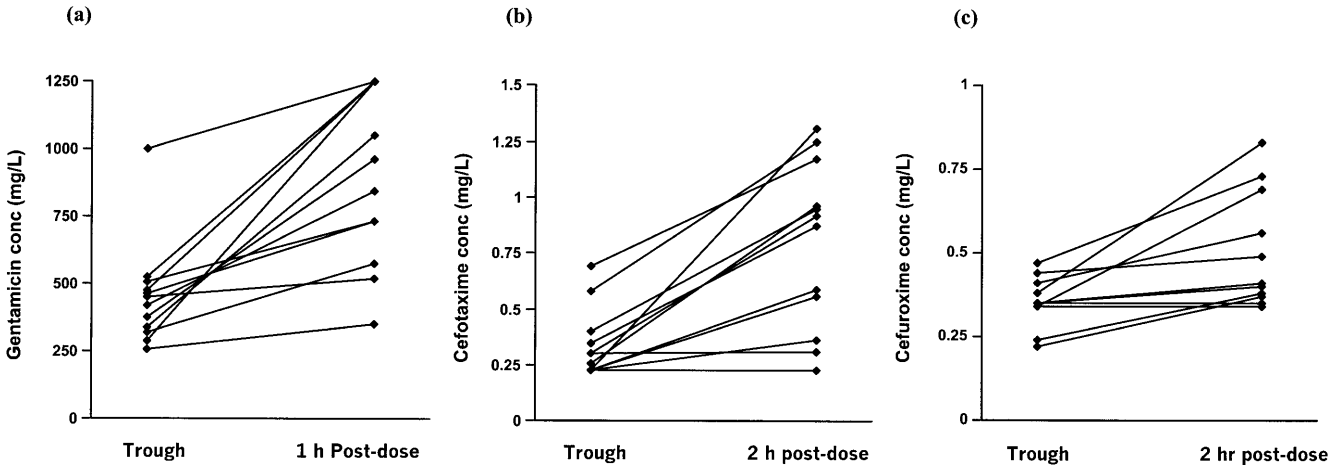

Fig. 2 Tracheal concentrations of **a** gentamicin, **b** cefotaxime and **c** cefuroxime in ventilated intensive care patients

sistant to the actions of antibiotics precedes colonisation of the airways with planktonic micro-organisms sensitive to antimicrobials. Aggregates of ET biofilm may become detached from the main body of biofilm by suction catheter and disseminate towards the lower respiratory tract, giving rise to infection [14].

This investigation demonstrates that nebulised gentamicin is effective in preventing the formation of biofilm composed of micro-organisms capable of causing VAP, as evidenced by the absence of recovered pathogens on ETs. Microbial biofilm recovered from five patients receiving gentamicin was probably the result of colonisation by oropharyngeal flora. The efficacy of this regimen may be attributed to the very high concentrations of gentamicin which can be achieved by this route of delivery. Despite these high tracheal concentrations, previous reports have shown that gentamicin undergoes minimal systemic absorption when delivered by nebuliser, is well tolerated by patients [10] and impacts positively on clinical and inflammatory airways indicia [11].

Endotracheal tubes from patients who received cephalosporins demonstrated considerable biofilm development. Because of their poor penetration into pulmonary tissue [15], these cephalosporins do not attain sufficient concentrations in pulmonary secretions to prevent the formation of ET biofilm. The relative lack of Gram-positive activity associated with cefotaxime is highlighted by colonisation of four ET tubes with *S. aureus*, in addition to some Gram-negative micro-organisms. In contrast, colonisation of ETs from patients receiving cefuroxime was predominantly Gram-negative, there being an absence of recovered *S. aureus*. As expected, enterococci, which have gained recognition as important causative micro-organisms of nosocomial infection, were resistant to both cefotaxime and cefuroxime, with MICs in excess of 1024 mg/l.

The sub-inhibitory tracheal concentrations of cephalosporins reported in this paper have important implications for device-related infection. For example, pathogens within a biofilm are less susceptible to the action of antibiotics than their free-floating (planktonic) counterparts and therefore will persist on the surface of the device despite antibiotic therapy [8]. Such concentrations will also promote microbial resistance which is quite distinct from the lack of susceptibility associated with the biofilm growth mode. Furthermore, sub-inhibitory concentrations of cephalosporins have been shown to promote microbial adherence to implanted devices [16].

The overuse of antibiotics in ICUs is well documented and the efficacy of antibiotic prophylaxis for VAP remains controversial [17]. Experience with other topical prophylaxis regimens, such as selective decontamination of the digestive tract (SDD), suggests its use can reduce VAP and associated mortality in certain patient groups, for example, those with trauma or who are immunosuppressed [18]. One variation on this approach has been to instil colistin solution, by syringe, into the tracheostomy tube as part of an SDD regimen, a regimen which was effective in reducing the incidence of VAP [19]. Nebulised gentamicin may offer a less expensive and less labour-intensive alternative to these regimens. It is the first regimen we have encountered which is effective in preventing the formation of ET biofilm by pathogens capable of causing VAP because of the high luminal concentrations. It may, therefore, be effective in reducing the incidence of VAP in high-risk patient groups. To this extent, the regimen warrants further clinical investigation for efficacy. In our unit bacterial surveillance has shown, over a 3 year period, that there has been no increase in the rate of Gram-negative resistance. Some resistant strains of *S. aureus* have been noted, but these were as contaminants rather than pathogens in patients with VAP.

Nonetheless, there is an increasing trend to minimise patient exposure to antibiotics [17, 18]. Within the context of medical devices, this can be achieved by either chemically bonding antibiotic to the surface of the de-

vice, or incorporating antibiotic into the constituent biomaterials of the device itself. The concept has been employed successfully in a wide variety of materials with reports of reduced microbial adhesion to, for example, tobramycin-impregnated PMMA bone cement [20], rifampicin-loaded silicone CSF shunts [21], polyurethane central venous catheters containing chlorhexidine and silver sulphadiazine [22], penicillin-releasing silicone CAPD catheters [23] and methacrylate dental resin incorporating antimicrobial agents [24]. Based on results from this study, high concentrations of antibiotic on the ET luminal surface, achieved either by nebuliser or ET surface modification [25], would be expected to prevent biofilm formation on the ET and therefore may have a role in reducing the incidence of VAP, while minimising patient exposure to systemic antibiotic.

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