Respiratory pathogen colonisation of dental plaque, the lower airways and endotracheal tube biofilms during mechanical ventilation

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Abstract:

Purpose: In mechanically ventilated patients, the endotracheal tube is an essential interface between the patient and ventilator, but inadvertently it also facilitates the development of ventilator-associated pneumonia (VAP) by subverting pulmonary host defense. A number of investigations suggest that bacteria colonising the oral cavity may be important in the aetiology of VAP. The present study evaluated microbial changes that occurred in dental plaque and lower airways of 107 critically ill mechanically ventilated patients. **Materials and Methods:** Dental plaque and lower airways fluid was collected during the course of mechanical ventilation, with additional samples of dental plaque obtained during the entirety of patients' hospital stay. **Results**: A 'microbial shift' occurred in dental plaque, with colonisation by potential VAP pathogens, namely, S*taphylococcus aureus* and *Pseudomonas aeruginosa* in 35 patients. Post-extubation analyses revealed that 70% and 55% of patients whose dental plaque included *S. aureus* and *P. aeruginosa,* respectively, reverted back to having a predominantly normal oral microbiota. Respiratory pathogens were also isolates from the lower airways and within the endotracheal tube biofilms. **Conclusions:** To the best of our knowledge, this is the largest study to date exploring oral microbial changes during both mechanical ventilation and following recovery from critical illness. Based on these findings, it was apparent that during mechanical ventilation, dental plaque represents a source of potential VAP pathogens.

Introduction

In mechanically ventilated patients the endotracheal tube is an essential interface between the lungs and the ventilator. Unfortunately, the presence of an endotracheal tube also impairs pulmonary host defenses and promotes ventilator-associated pneumonia (VAP) through supporting biofilm formation within its inner lumen [1,2]. In addition, the endotracheal tube and incomplete mouth closing will alter the oral microenvironment. VAP is the most common nosocomial infection in critical care with a prevalence of approximately 15% and prognosis is negatively influenced with involvement of multidrug resistant pathogen biofilms [3,4]. The endotracheal tube biofilm may serve as a reservoir of respiratory pathogens that are largely protected from host defense mechanisms. In recent years, studies into the origin of VAP causing microorganisms have primarily focused on oropharyngeal sites rather than the gastro-intestinal tract [5-8]. As a consequence, a number of recent strategies aimed at preventing VAP have sought to target the oral microbiome [9,10].

Dental plaque was initially considered to be a bacterial construct (*i.e*. a biofilm) in the 1970s [11]. Dental plaque harbors an estimated 500 different bacterial species with variation in microbial composition occurring between and along the teeth [12]. *Streptococcus* species are recognised as primary pioneer colonisers of teeth and are initiators of dental plaque development [13]. Lazarevic et al. analysed the oral microbiome using molecular methods and reported that up to 70% of sequences belonged to the *Streptococcus* and *Neisseria* genera [14]. Saliva plays an important role in modulating dental plaque formation [15, 16]. Glycoproteins and proline-rich proteins (PRPs) in saliva will adsorb to tooth surfaces generating an enamel pellicle allowing bacteria to adhere [17].

The oral microbiome could promote VAP in several ways [18]. Firstly, during mechanical ventilation rapid colonisation by potential respiratory pathogens including *Pseudomonas, Klebsiella, Staphylococcus aureus* and *Acinetobacter* can occur and these bacteria may subsequently disseminate to the lung [8,19,20]. Secondly, commensal oral bacteria may actively promote

respiratory pathogen colonisation of the endotracheal lumen, and these bacteria may again translocate to the lower airways leading to VAP [1,21]. Biofilm-mediated infections are difficult to treat, as not only are the cells protected within the biofilm structure, but the microorganisms involved are also frequently inherently less susceptible to antimicrobial agents [22-24].

Colonisation of dental plaque by respiratory pathogens is important in VAP aetiology, and it is also known that for the majority of critically ill patients, oral hygiene frequently deteriorates during mechanical ventilation [25,26]. Furthermore, not all oral hygiene interventions appear effective at reducing VAP incidence [27,28] and a recent meta-analysis even suggested that accepted oral hygiene treatments, such as use of chlorhexidine may actually lead to harmful effects [29].

In order to deliver effective oral care to critically ill patients to reduce VAP, it is important to increase our understanding of the dynamics of the oral microbiome during mechanical ventilation and how this relates to contamination of both the ETT and lower airway. The current study examined the nature of microbial changes in dental plaque and the lower airway, during mechanical ventilation and, in contrast to previous studies, during the patient recovery after mechanical ventilation.

Materials and Methods

Methods

Patient recruitment

Ethical approval was obtained from the National Research Ethics Service (NRES) within the Research Ethics Committee (REC) for Wales (Ref: 13/WA/0039). In order for sufficient statistical power (>80%) to observe a 20% change in at least one phylum in microbial profiles and associated

downstream high-throughput techniques [30], the minimum number of participants required for the study was 101. Mechanically ventilated patients were eligible for inclusion in the study if they were aged >18 years, had >8 original teeth, their anticipated period of mechanical ventilation was >24h and their expected survival was >24 h. Informed consent for participation in the study was obtained from the next of kin and also taken from the patient if they recovered capacity.

Within 6h of critical care admission, a critical care mouth plan was completed to determine the level and frequency of oral care required. Oral care included toothbrushing 4 times a day with sterile water, moistening of the oral cavity and lips. Antiseptic mouthwashes were not used. Additional care was provided for denture wearers. VAP was diagnosed using the existing Patients with a clinical suspicion of VAP had a Clinical Pulmonary Infection Score (CPIS) calculated using parameters of temperature, white blood cell count, PaO_2/FIO_2 ratio, the presence of tracheal secretions and changes on chest radiograph. Quantitative microbiological culture (>10^3CFU/ml) of the lower airways by (bronchoalveolar lavage (BAL)/non-directed bronchoalveolar lavage (NBL) was undertaken if the CPIS score was >6BAL was performed bronchoscopically by an attending clinician, whilst NBLs were undertaken by the bedside nurse inserting a suction catheter through the catheter mount into the lung parenchyma and flushing and withdrawing sterile saline [32-36].

Decayed, missing and filled teeth (DMFT) score

The DMFT score was recorded (by a dental professional). The DMFT score is a measurement of dental caries status and is therefore an indicator of prior longer-term oral hygiene levels [31]. Each incidence of a tooth recorded as decayed, missing or filled results in a score of 1 to generate a score of 0-28, with higher scores representing poor oral health. Final DMFT scores can therefore range between 0-28. DMFT scores, although not reflective of the remaining dentition, may indicate prior differences in oral hygiene

maintenance within the patient cohort and therefore the level of risk for dental plaque changes.

Dental plaque collection

Subgingival and supragingival plaque was collected using paper points (QED size 40) and dental examination kits (Minerva Dental) [37]. Collection was performed on 3 occasions during the first week of admission to critical care and then weekly. The fist sample was collected within 24 h the start of mechanical ventilation. A total of 9 paper points, sampling 3 teeth per area (front, middle and back) were used per collection. In cases where the patient did not have sufficient teeth for the above protocol, plaque was taken from the closest areas. Plaque specimens were suspended in transport medium [38] and processed using microbial culture on the day of collection.

Collection of subglottic aspirations, non-directed bronchoalveolar lavages (NBLs), bronchoalveolar lavages (BALs) and endotracheal tubes (ETTs)

Subglottic secretions were collected through a subglottic port in the ETT using a syringe and transferred into sterile universals. Subglottic aspirates and NBLs were aseptically transferred into universal containers and endotracheal tubes were collected and placed in sterile bags when available for transport to the microbiology laboratory.

Identification of respiratory pathogens

Clinical specimens were processed within a Class II safety cabinet. Dental plaque was vortex-mixed and spread-plated on to appropriate selective agar media for detection of *Staphylococcus aureus* (MSB; Mannitol Salt Agar, MSA) and *Pseudomonas aeruginosa* (Pseudomonas agar base; Lab M), which are prevalent VAP pathogens [39-41]. Agar media were incubated at 37°C under aerobic conditions for 5 days.

NBLs and BALs were centrifuged for 3 min at 10,000*g,* and the pellet re-suspended in 1 ml of PBS. A 50-µl volume was spread-plated on the previously described selective agar. Growth of *P. aeruginosa* and *S. aureus* was

recorded, and colonies of presumptive respiratory pathogens identified by biochemical testing. *Staphylococcus aureus* colonies were sub-cultured on MSA agar for 18-24 h at 37°C and tested for catalase and coagulase activity [42]. Colonies of *P. aeruginosa* were sub-cultured on *Pseudomonas* agar for 18-24 h at 37°C and tested for oxidase activity.

Definitive identification of *S. aureus* and *P. aeruginosa* was by species-specific PCR (Table 1) [43,44]. DNA extraction employed a commercially available DNA extraction kit (Qiagen). PCR was performed in a total reaction volume of 50 µl containing 2 µl of DNA template. Thermal cycling parameters for *S. aureus* detection were an initial 5 min at 94ºC, followed by 35 cycles of 94ºC for 40 s, 50ºC for 40 s and 72ºC for 1 min, with a final elongation step of 72ºC for 10 min. For *P. aeruginosa* PCR, there was an initial denaturation step of 95ºC followed by 35 cycles of 94ºC for 45 s, 58.4ºC for 45 s and 72ºC for 1 min ending with 5 min at 72ºC.

Antimicrobial susceptibility profiling

Staphylococcus aureus and *P. aeruginosa* were cultured on Mueller Hinton agar at 37°C for 18-24 h. A 0.5 McFarland standard (10^8 cells/ ml) was prepared to create an inoculum for antimicrobial sensitivity testing. A sterile swab was used to homogenously inoculate the 0.5 McFarland standard across the agar. Cefoxitin discs were used to identify MRSA, whilst sensitivity profiles of *P. aeruginosa* and *S. aureus* were tested against 6-12 antimicrobials discs (selected based on previous administration to patients and according to frequent antibiotics used for these microorganisms). Agars were incubated for 18-24 h at 37°C and subsequent zones of inhibition (ZOI) measured and categorisation of isolate susceptibility (sensitive, resistant and intermediate resistant) was done according to British Society for Antimicrobial Chemotherapy (BSAC) guidelines. For analysis, the isolate antimicrobial sensitivities were grouped into clinical site origin.

Imaging of endotracheal tube biofilms

A 0.5-cm section of an ETT was placed in 2 ml of 10% (v/v) formalin for 24 h, processed in embedded wax and sectioned to 20 µm. Peptide nucleic acid

probes (PNA; 100 µmol; Panagene; Table 2) were prepared in hybridisation solution (10% (w/v) dextran sulphate, 10 mM NaCl, 30% (w/v) formamide, 0.1% (w/v) sodium pyrophosphate, 0.2% (w/v) polyvinylpyrolidone, 0.2% (w/v) ficol, 5 mM disodium EDTA, 50 mM Tris HCl and 0.2% Triton-X at pH 7.5) [45].

Processing involved enzymatic pre-treatment to promote access of the biofilm to PNA probe hybridisation. A 50-µl volume of lysostaphin was added to the section which was incubated at 20°C for 30 min. A 100-µl volume of lysozyme (10 mg/ml) was then added followed by incubation at 37°C for 30 min. A 100-µl volume of PNA probe (300 nM for all probes with exception of the *S. aureus*-specific probe which was at 450 nM) was added and incubated at 55°C for 90 min. Sections were flooded with 2 ml of wash solution (5 mM Tris, 15 mM NaCl and 1% (v/v) Triton X-100 at pH 10) and incubated at 55°C for 15 min [45] prior to mounting under coverslips with Vectashield™ (Vectorlabs). Controls devoid of probe were included.

Sections were imaged using a Leica TCS SP2 AOBS spectral confocal microscope (Leica, Heidelberg, Germany) and appropriate excitation and emission settings for FITC (ex max 494nm; em max 518nm); Cy 3 (ex max 550nm; em max 570nm) and Cy 5 (ex max 650nm; em max 670nm). Micrographs were presented as image overlays of confocal fluorescence (colour) superimposed upon Nomarski differential interference contrast (greyscale).

Statistical analysis

Where appropriate, statistical analysis (t test sampling) was performed using IBM SPSS v20.

Results

Patient recruitment and demographics

A total of 1016 patients were screened over 14 months. Of these, 5 patients were <18 years, 20 patients had <8 teeth, 210 patients were anticipated to be mechanically ventilated for <24h, 439 patients were not mechanically ventilated and 232 patients could not be consented. A total of 107 patients (65 male and 42 female; mean age 54) met the inclusion criteria and were recruited following receipt of informed consent. The median duration of mechanical ventilation was 7 days.

The study was performed in a single adult critical care unit. Patients were recruited with various health backgrounds for admission to critical care. Patient demographics and clinical measurements are presented in table 3.
There was a lower mean age (39 y) for acute/ poly-trauma (n=12), compared to respiratory failure (n=30; mean age of 60 y). The mean age of patients admitted/receiving mechanical ventilation following an out of hospital cardiac arrest (OOHCA) was 60 y (n=11). A total of 77 (71%) of mechanically ventilated patients received at least one antibiotic, and just under half of patients (49%) received >2 different antibiotics during the course of mechanical ventilation. Over 30 antibiotics were administered to patients during the clinical study (table 3). Antifungals (including nystatin and fluconazole) were also administered to mechanically ventilated patients (n=12). Of 107 patients, DMFT indices were obtained for 97 and these scores increased with age; a score >10 was typically recorded for patients >40 years of age.

Microbiological analysis during mechanical ventilation

A total of 848 dental plaque samples were obtained from 107 mechanically ventilated patients. Of these, 592 were collected during mechanical ventilation, with a mean number of plaque samples of 5 per patient. At least one dental plaque specimen was colonised with either *S. aureus* (43 patients) or *P. aeruginosa* (23 patients) during mechanical ventilation. Co-isolation of *S. aureus* and *P. aeruginosa* occurred for 10 patients. Of 43 patients who were culture positive for *S. aureus* during mechanical ventilation, 21 (48%) were

culture negative for *S. aureus* at the time of intubation. A total of 23 patients were culture positive for *P. aeruginosa* during mechanical ventilation, with 18 (78%) patients culture negative for this species at the time of intubation (Figure 1). The dental plaque of 35 patients out of 107 (33%) therefore exhibited a change in microbial composition to incorporate at least one of the targeted respiratory pathogens.

Staphylococcus aureus was detected in the lower airways of 37 patients, and predominately occurred with concurrent dental plaque colonisation. The subglottic secretions of 14 patients and 4 patients contained *S. aureus* and *P. aeruginosa*, respectively. Co-colonisation of *P. aeruginosa* from both dental plaque and lower airway specimens was higher than for *S. aureus*. Twenty nine patients were culture positive for *P. aeruginosa,* and 23 of these were positive from dental plaque culture. ETT biofilm imaging using PNA-CLSM facilitated detection and spatial location of targeted species in the ETT biofilm (Figure 2).

Forty-one patients were clinically diagnosed and treated for VAP during the study. This apparent high VAP rate can be related to the patient cohort as many had prolonged ventilation (13 ventilated between 5 – 7 d and 57 ventilated >7 d). Of these patients, 18 had respiratory pathogens within dental plaque during mechanical ventilation, and 24 patients were colonised with the same respiratory pathogen within the dental plaque and the lower airways at any time point. In addition 9 patients clinically treated for VAP had respiratory pathogens in their dental plaque from the start and over the course of mechanical ventilation.

Antimicrobial sensitivity of *S. aureus* and *P. aeruginosa* isolates

The majority (>70%) of tested isolates from all sites were susceptible to the antibiotics tested. A total of 114 isolates of *S. aureus* (table 4) were recovered both during mechanical ventilation and into the recovery period. Where differences were observed in sensitivities, *S. aureus* isolates with antibiotic resistance profiles were most frequently isolated from subglottic secretions

and tended to more frequently exhibit resistance to erythromycin, penicillin and cefepime. The majority of *S. aureus* were sensitive to cefoxitin and ceftazidime irrespective of origin. Of the 56 *P. aeruginosa* isolates, 35 were from dental plaque. Antimicrobial resistance patterns for all tested antibiotics ranges between 2% (ciprofloxacin) to 23% of strains (meropenem) (table 5). Although only 5 *P. aeruginosa* isolates were recovered from endotracheal tube sections and one of these exhibited the most resistant profile across all antibiotics tested. *P. aeruginosa* isolates exhibited the greatest sensitivity to Tobramycin with antibiotic susceptibilities ranging from 80% of isolates sensitive to all antimicrobials in the ETTs, to 97.1% in the dental plaque.

Antimicrobial sensitivity patterns for recovered isolates of *P. aeruginosa* between dental plaque, the lower airways and endotracheal tube biofilms were largely similar for 7 out of 10 patients. Similarly, for recovered isolates of *S. aureus* between dental plaque, the lower airways and endotracheal tube biofilms, antimicrobial sensitivity patterns were related for 21 out of 30 patients. (All individual isolate sensitivities are shown within the supplementary material).

In the context of this study, an MDR pathogen was defined as a pathogen exhibiting a resistant profile (In terms of ZOI according to BSAC guidelines) to at least three antibiotics. A total of 3 patients were colonized with MDR *P. aeruginosa* and 21 patients were colonized with an MDR *S. aureus* (8 patients colonized with MRSA as detected by resistance to cefoxitin with a ZOI <20mm).

Dental plaque analysis after endotracheal tube extubation

A total of 256 dental plaque samples were collected during the recovery period with 88 collected within 1 week post-endotracheal tube extubation, 66 during week 2 post-extubation, 43 collected 3 weeks post-extubation and a further 59 were collected >1 month post-extubation. For a total of 31 patients, dental plaque was not collected due to either patient withdrawal upon recovery, or death. Out of the 35 patients that exhibited microbial changes

during mechanical ventilation, analysis of post-extubation dental plaque was completed for 27 patients.

These analyses allowed an assessment of persistence of respiratory pathogens in patients' dental plaque. In patients where dental plaque was colonised with *S. aureus,* 71% reverted back to a predominantly normal oral microbiota, devoid of *S. aureus* colonisation after extubation. Similarly for patients whose plaque was colonised by *P. aeruginosa,* 55% became culture negative for *P. aeruginosa* after extubation. A bar graph (Figure 1) compares the colonisation of respiratory pathogens during mechanical ventilation and the recovery period, highlighting reversed-microbial changes during the recovery period. Readmission rates to critical care were similar for patients exhibiting a reverse-microbial change (11.1%) and those patients harboring pathogens within their dental plaque during the recovery period (10%).

Discussion

VAP is an important hospital acquired infection in critically ill patients [4] and is associated with increased mortality, duration of stay and cost [46]. Prevention of VAP is vital, and an important facet in the development of appropriate preventative strategies is a better understanding of the aetiology and pathogenesis of VAP [47,48].

The microbiome of the oral cavity is both highly diverse and dynamic, primarily because of the wide range of microbial habitats that exist in the mouth and the fluctuations that can arise in these environments due to changes in diet, salivary flow and oral hygiene interventions [49-54]. Unsurprisingly, since the oral cavity is directly linked to the lower airways, associations between oral microbiology and respiratory infections are frequently made. In the case of VAP, it has been suggested that oral microorganisms could promote colonisation of dental plaque and endotracheal biofilms by potential respiratory pathogens, or may directly cause VAP themselves [55]. Carrilho-Neto et al, showed a reduction in oral hygiene for the majority of hospitalized patients, reporting a positive correlation between dental plaque index and gingival index [56]. Gingival

inflammation caused by poor oral hygiene in intubated patients may also drive inflammation within the lungs [56-58]. The primary objective of this study was to determine the colonisation dynamics for key microbial species at defined sites in critically ill patients undergoing mechanical ventilation. Although previous studies have examined colonisation with potential respiratory pathogens following critical illness, this has only been over a short duration of ventilation following intubation.

An important finding of this study was that the composition of dental plaque in a significant proportion (approximately one third) of mechanically ventilated patients altered with inclusion of the potential respiratory pathogens *S. aureus* and *P. aeruginosa.* Importantly, these bacterial species may exhibit resistance to antibiotics, and are causative agents in up to 50% of VAP cases [39-41]. The presence of these targeted microorganisms in the endotracheal tube was also evident using the culture independent tool of PNA-FISH coupled with CLSM. Aggregates of respiratory pathogens were clearly evident using this approach. The displacement of respiratory pathogens to the lower airway would deliver infectious agents already within a biofilm phenotype and are therefore more resistant to host defence mechanisms and administered antimicrobials. Although only a small proportion of *S. aureus* isolates were reported as MRSA, ~25% isolates recovered from dental plaque were resistant to at least one antibiotic tested *in vitro* (tables 4 and 5.) When assessing antimicrobial resistant levels between isolates recovered within the dental plaque and the lower airways, the highest levels of resistance were recovered from outside of the oral cavity. Resistance rates were highest for *P. aeruginosa* within the ETT biofilm, and for *S. aureus* within subglottic secretions (table 4). This can imply higher rates of resistance within the airways, and if VAP were to develop in these patients then this could exacerbate the success of antimicrobial therapy.

Importantly, in the majority of patients where microbial changes occurred in the dental plaque, a reversal occurred once the patient was extubated, and this was most readily evident with *S. aureus* colonisation. A higher proportion of patients colonised with *P. aeruginosa* retained the respiratory pathogen

colonisation post extubation. Although most dental plaque communities reverted back to a phenotype without target respiratory pathogens within one week of extubation, the fact that some patients remained colonised with respiratory pathogens over a prolonged duration could represent a patient group at risk of subsequent hospital-acquired pneumonia.

Although not regarded as a normal inhabitant of the oral cavity, *S. aureus* has been detected within the dental plaque of debilitated or elderly individuals [59]. An observational study of hospitalized patients by Sachdev et al, (62% were not ventilated), revealed colonization rates of *S. aureus* at ~14% [26]. In the current study, the high incidence (43 of 107 patients) of *S. aureus* in dental plaque was nevertheless surprising, particularly as half of these patients did not have *S. aureus* in their dental plaque at the time of intubation. Similarly, dental plaque also became colonised with *P. aeruginosa* during mechanical ventilation, albeit at a lower incidence. As dental plaque is easily sampled, and is a less invasive procedure than a NBL or BAL, isolation of potential respiratory pathogens in dental plaque of patients with suspected VAP may enable targeted antimicrobial therapy and should be evaluated in future studies. In 6 mechanically ventilated patients, although *P. aeruginosa* was isolated within the lower airways there was no confirmation of *P. aeruginosa* via microbial culture within their dental plaque. Perhaps further investigation coupled with high-throughput technologies could elucidate whether *P. aeruginosa* could be detected if present in a much lower abundance. Furthermore, there is the potential for dental plaque analysis in guiding empiric therapy, however false negatives when relating to the occurrence of respiratory pathogens in the lower airways can occur.

One of the main limitations of the current study is that target pathogens were limited to *S. aureus* and *P. aeruginosa* by culture specific methods. Whilst additional respiratory pathogens were not assessed in this study, others have found that *E. coli, Klebsiella* species and *Acinetobacter* species may also colonise dental plaque and endotracheal tubes during mechanical ventilation [2]. Furthermore we have demonstrated considerable microbial diversity and colonisation of plaque with potentially pathogenic bacteria using non-culture

techniques such as community profiling by high throughput sequencing [30]. The reason(s) why such microbial changes occur in dental plaque remain unclear, but are likely linked to local environmental changes in the mouth. These may include plaque accumulation and gingival inflammation from inadequate delivery of oral care during mechanical ventilation, perturbations of salivary composition and reduced salivary flow as a consequence of incomplete mouth closure, or following receipt of drugs [60].

This is to our knowledge, the first study in critically ill mechanically ventilated patients that has sequentially assessed the dental microflora over a prolonged duration and shown a decrease in respiratory pathogen colonisation of dental plaque in some patients during recovery from critical illness. Once elucidated, the reasons why the dental plaque of some patients' begins to revert back to a profile of microbes without respiratory pathogens (identified during mechanical ventilation), potentially offers new preventative strategies for VAP. Whether those patients who have persistent colonisation with respiratory pathogens despite recovery from critical illness are at increased risk of hospital acquired infection needs to be evaluated in larger adequately powered studies. It was evident from this present study that microbial changes occur in the dental plaque of mechanically ventilated patients and these include colonisation by respiratory pathogens. The presence of respiratory pathogens in dental plaque is a risk factor for VAP. Emphasising the importance of maintaining oral hygiene during mechanical ventilation, may actually limit this reservoir of respiratory pathogens within the dental plaque of mechanically ventilated patients.

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Species	Target Gene	Primers	Amplicon size	Reference
S aureus	Vick	vicK1: 5'-CTA ATA CTG AAA GTG AGA AAC GTA-3' vicK2: 5'-TCC TGC ACA ATC GTA CTA AA-3'	289bp	32
P. aeruginosa	*ecf*X	Ps.aeru_ECF1: 5'-ATG GAT GAG CGC TTC CGT G -3' Ps.aeru_ECF2: 5'-TCA TCC TTC GCC TCC CTG -3'	528 bp	33

Table 1 - PCR primers for identification of *S. aureus* and *P. aeruginosa.*

Probe	Nucleotide sequence (5'-3')	Fluorescent marker (N-terminal)	final probe concentration (nM)
Bacterial Universal	CTGCCTCCCGTAGGA	Cy3-00-	300
Pseudomonas aeruginosa	AACTTGCTGAACCAC	FITC-00-	300
Staphylococcus aureus	GCTTCTCGTCCGTTC	Cy5-00-	450
Candida albicans	ACAGCAGAAGCCGTG	FITC-00-	300

Table 2 - Species-specific PNA probes and associated fluorescent labels

Recruited patients	Patient NO.	Gender	Age	Admission details (reason for MV)	DFMT	Antibiotic use
1	PN001	M	51	Respiratory failure	8	Clindomicin, Meropenem
2	PN002	M	33	Stroke/brain injury/seizures	4	-
3	PN003	M	31	Overdose/suicide attempt	9	Co-amoxiclav
4	PN004	F	55	Stroke/brain injury/seizures	19	Cefuroxime
5	PN005	M	62	Stroke/brain injury/seizures	21	Cefuxome (in theatre)
6	PN006	F	34	Respiratory failure	0	Meropenem, Gentimicin, Co-tremoxyle
7	PN007	F	54	Stroke/brain injury/seizures	11	Cefuroxime
8	PN008	M	63	Respiratory failure	12	Clarthromycin, co-amoxiclav
9	PN009	F	77	Other	23	Meropenem, Tazocin
10	PN010	F	30	Stroke/brain injury/seizures	-	Trimethroprim
11	PN011	F	73	Respiratory failure	16	Augmentin Tazocin, Clarithromycin
12	PN012	M	68	Respiratory failure	13	Tazocin
13	PN013	F	50	Stroke/brain injury/seizures	14	-
14	PN014	M	44	Respiratory failure	11	Meropenem, Tazocin
15	PN015	F	49	Stroke/brain injury/seizures	11	Cefuroxime
16	PN016	M	28	Other	4	Clindomicin, Meropenem
17	PN017	F	51	OOHCA	23	-
18	PN018	M	39	Overdose/suicide attempt	-	-
19	PN019	M	69	Poly-trauma	22	Teicopleinin, Cefuroxime, Tobramycin, Erythromycin
20	PN020	F	29	Poly-trauma	3	Tazocin,
21	PN021	F	18	Stroke/brain injury/seizures	0	Aciclovir
22	PN022	M	72	OOHCA	12	Tazocin
23	PN023	M	19	Stroke/brain injury/seizures	5	Tazocin, Clarithomycin
24	PN024	M	40	Stroke/brain injury/seizures	14	-

25	PN025	F	27	Stroke/brain injury/seizures	4	-
26	PN026	F	63	General surgery - Stomach	-	Meropenem
27	PN027	M	41	Stroke/brain injury/seizures	5	-
28	PN028	F	55	Respiratory failure	15	-
29	PN029	M	76	Respiratory failure	15	Co-amoxiclav, Tazocin
30	PN030	F	58	Stroke/brain injury/seizures	19	Ciprofloxacin, Metronizadole, Gentamycin
31	PN031	F	31	Other	-	Meropenem,
32	PN032	M	67	Stroke/brain injury/seizures	11	Augmentin, Tazocin, Clarithromycin
33	PN033	F	85	Respiratory failure	17	Amoxicillin, Co-amoxiclav, Clarithromycin, Tazocin
34	PN034	M	57	Other	18	Cefuroxime, Metronidazole, Ciprofloxacin, Gentamycin
35	PN035	M	58	OOHCA	19	Tazocin
36	PN036	M	60	OOHCA	-	
37	PN037	M	37	Stroke/brain injury/seizures	15	Ceftrixone, Meropenem, Ciprofloxacin
38	PN038	M	37	Stroke/brain injury/seizures	0	Meropenem, Ciprofloxacin, Ceftriaxome
39	PN039	F	86	Respiratory failure	17	Co-amoxiclav, Tazocin, Clarithromycin
40	PN040	F	60	Respiratory failure	21	-
41	PN041	F	58	Respiratory failure	26	-
42	PN042	M	74	Stroke/brain injury/seizures	15	-
43	PN043	M	59	Stroke/brain injury/seizures	6	-
44	PN045	F	22	Stroke/brain injury/seizures	-	Tazocin
45	PN046	M	48	OOHCA	12	-
46	PN047	M	53	Stroke/brain injury/seizures	12	-
47	PN048	M	57	Stroke/brain injury/seizures	7	Cefuroxime
48	PN049	F	75	General surgery - Stomach	16	Gentamycin, Tazocin
49	PN050	M	39	Stroke/brain injury/seizures	11	-
50	PN051	F	73	Stroke/brain injury/seizures	8	Cefalexin, Meropenem
51	PN052	M	65	Other	16	-

52	PN053	F	66	Respiratory failure	11	Vancomycin, Tazocin
53	PN054	M	66	Other	13	
54	PN055	F	60	Other	8	Meropenem, Vancomycin
55	PN056	F	49	Stroke/brain injury/seizures	17	-
56	PN057	M	53	Respiratory failure	11	-
57	PN058	F	38	Stroke/brain injury/seizures	6	Meropenem
58	PN059	M	86	General surgery - Stomach	28	Tazocin
59	PN060	M	39	Stroke/brain injury/seizures	9	Cefuroxime, Co-amoxicillin
60	PN061	M	36	Poly-trauma	14	Tobramycin, Colomycin, Cefuroxime
61	PN062	M	21	Poly-trauma	1	Tobramycin, SDD oral paste, Colomycin, Cefotaxime,
62	PN063	M	58	Respiratory failure	-	Amikacin, Tazocin, Clarithromycin
63	PN064	M	44	OOHCA	23	-
64	PN065	F	42	General surgery - Stomach	13	Gentamycin, Augmentin, Metronidazole, Tazocin,
65	PN066	F	73	Respiratory failure	0	Augmentin, Tazocin, Meropenem
66	PN067	M	68	Respiratory failure	19	Meropenem
67	PN068	M	52	OOHCA	8	-
68	PN069	F	82	General surgery - Stomach	20	-
69	PN070	M	18	Poly-trauma	2	Cefotaxime, (SDD regime Nystatin, Colomycin), Tobramycin
70	PN071	M	26	Poly-trauma	7	Augmentin, SDD: Nystatin, Colomycin, Cefuitoxime
71	PN072	M	67	Respiratory failure	10	Tazocin
72	PN073	M	59	Other	19	-
73	PN074	F	51	Stroke/brain injury/seizures	17	Rifampicin, Meropenem, Vancomycin
74	PN075	M	75	Stroke/brain injury/seizures	20	Cefotaxime, Amoxicillin, Meropenem, Vancoymcin, Gentamycin
75	PN076	M	49	Respiratory failure	16	Tazocin, Clarithromycin
76	PN077	M	70	Stroke/brain injury/seizures	18	Cefuroxime
77	PN078	M	75	Respiratory failure	24	cotrimazole cream, Meropenem, Vancomycin
78	PN079	F	51	Respiratory failure	-	Tazocin, Trimethroprim

79	PN080	F	71	Respiratory failure	6	Meropenem
80	PN081	M	74	Other	18	Tazocin
81	PN082	F	45	Stroke/brain injury/seizures	1	Gentamicin
82	PN083	M	47	Respiratory failure	1	Augmentin, Clarithromycin
83	PN084	M	58	Poly-trauma	17	Augmentin, SDD: Colomycin and Tobramycin, Teicoplanin
84	PN085	F	75	Respiratory failure	14	Meropenem
85	PN086	M	74	Stroke/brain injury/seizures	5	
86	PN087	M	80	OOHCA	11	
87	PN088	F	40	Dental/Oral cavity	22	Meropenem, Clindamycin, Metronidazole
88	PN089	M	38	Poly-trauma	15	Co-amoxiclav, Tobramycin, (SDD: Colistin + Nystatin)
89	PN090	M	66	Respiratory failure	17	Tazocin, Co-trimoxizole
90	PN092	M	55	Stroke/brain injury/seizures	13	Tazocin
91	PN093	F	42	Stroke/brain injury/seizures	7	Meropenem, Ceftriaxone, Rifampicin, Vancomycin
92	PN094	M	60	Respiratory failure	13	Co-amoxiclav, Clarithromycin
93	PN095	M	41	Stroke/brain injury/seizures	9	-
94	PN096	F	27	Other	1	Amoxicillin, Ceftriaxone
95	PN097	F	77	Poly-trauma	22	Colomycin, Tobramycin, Cefotaxime
96	PN099	M	58	Other	9	Rifampicin, Isoniazid, Pyrazinamide, Ethambutol
97	PN100	M	26	Overdose/suicide attempt	6	Clarythromcin, Cefotaxime
98	PN101	M	29	Respiratory failure	25	Meropenem
99	PN102	M	30	Respiratory failure	8	Co-amoxiclav, Clarithromycin, Co-trimoxazole
100	PN103	F	80	Respiratory failure	-	Augmentin, Co-amoxiclav, Clarithryomcin
101	PN104	M	56	OOHCA	18	-
102	PN105	M	67	OOHCA	14	-
103	PN106	F	60	Respiratory failure	19	Amoxicilin
104	PN107	F	47	Stroke/brain injury/seizures	12	-
105	PN108	M	74	OOHCA	-	-

106	PN109	M		Respiratory failure	7	Tazocin
107	PN110	M	60	Other		Metronidazole, Cefotaxime

Table 3 - Patient demographics: age, gender, DMFT scores, admission details (primary reason for mechanical ventilation) and antibiotic administration

	114 Isolates of *S. aureus*					
	Cefepime	Cefoxitin	Ceftazidime	Fusidic Acid	Gentamicin	Meropenem
Sensitive %	65	88	88	89	82	86
Intermediate %	20	0	2	4	13	6
Resistant %	15	12	11	7	4	8

	Ciprofloxacin	Clindamycin	Erythromycin	Penicillin	Tobramycin	Vancomycin
Sensitive %	86	61	62	55	79	88
Intermediate %	4	27	16	31	15	1
Resistant %	11	12	22	14	6	11

Table 4 - Antimicrobial sensitivities for *S. aureus* isolates

	56 Isolates of *P. aeruginosa*						
	Ceftazidime	Ciprofloxacin	Gentamicin	Meropenem	Piperacillin	Piperacillin-Tazobactam	Tobramycin
Resistant %	8.8	1.8	1.8	22.8	15.8	10.5	1.8
Sensitive %	82.5	87.7	66.7	64.9	40.4	63.2	96.5
Intermediate %	8.8	10.5	31.6	12.3	43.9	26.3	1.8

Table 5 - Antimicrobial sensitivities for *P. aeruginosa* isolates

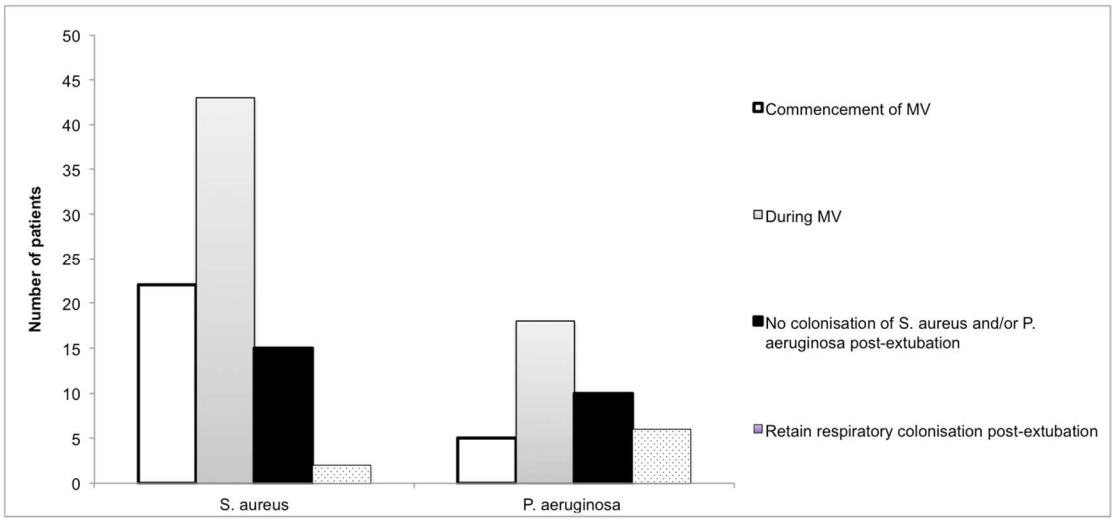

Figure 1 - Microbial colonisation of respiratory pathogens during endotracheal intubation and analysis during the recovery period (up to 8 weeks post ETT-extubation).

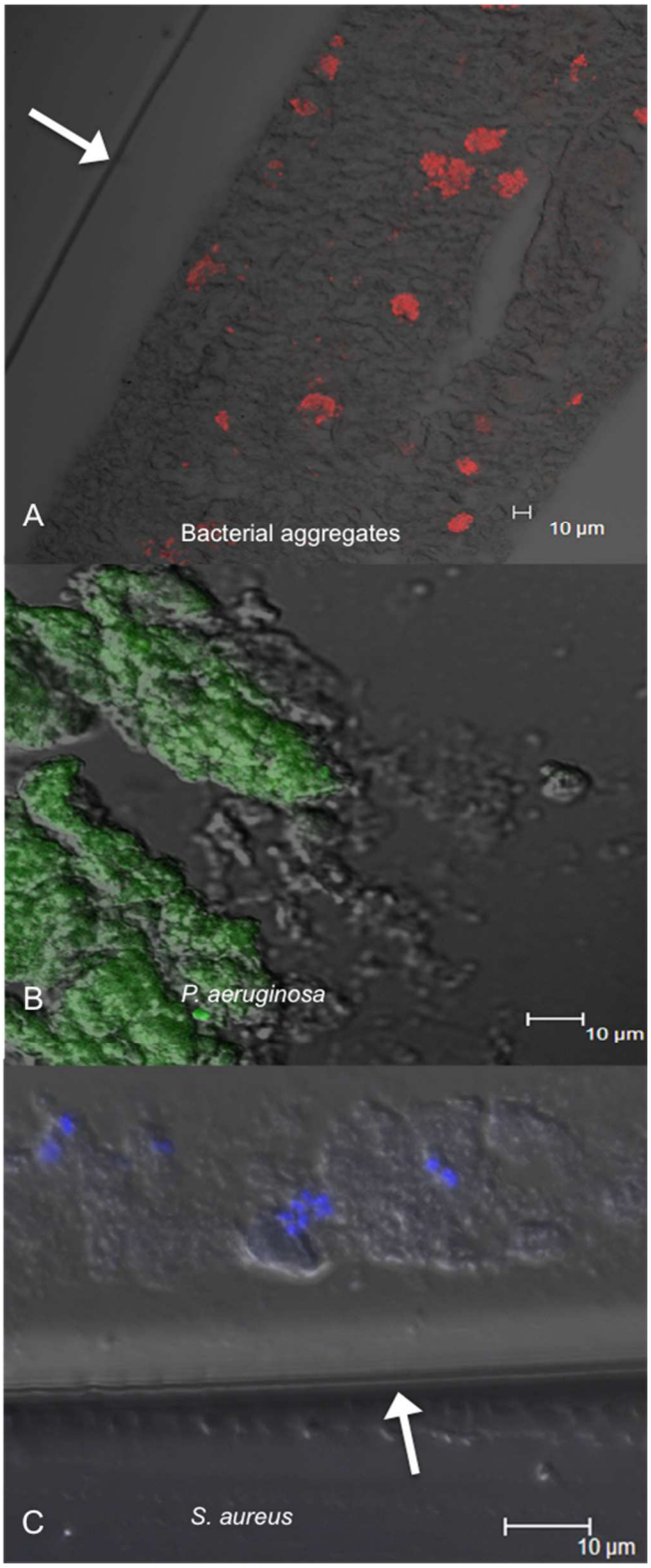

Figure 2 - Micrographs of endotracheal tube (ETT) biofilm obtained by confocal laser scanning microscopy (CLSM). All micrographs show a confocal fluorescence image superimposed (colour) upon a Nomarski differential interference (greyscale) A) Aggregates of bacteria hybridised with the universal bacterial Peptide Nucleic Acid (PNA) probe labeled with Cy-3

(red); B) *Pseudomonas aeruginosa* hybridised with species specific FITC labelled PNA probe (green); C) *Staphylococcus aureus* hybridised with species specific PNA probe conjugated with Cy-5 (blue). Where possible, the edge of the ETT section is arrowed.

Patient		Antibiotic											
Patient	**Sample**	**Cefepime**	**Cefoxitin**	**Ceftazidime**	**Ciprofloxacin**	**Clindamycin**	**Erythromycin**	**Fusidic Acid**	**Gentamicin**	**Meropenem**	**Penicillin**	**Tobramycin**	**Vancomycin**
PN004	SUB	25	0	20	35	0	0	0	26	26	0	22	0
PN005	DP	28	29	16	28	0	0	30	24	34	16	22	16
PN005	DP	27	29	19	28	0	0	36	24	38	17	23	16
PN007	DP	27	30	20	20	27	26	30	22	34	40	22	17
PN007	DP	28	29	18	21	36	25	36	22	36	16	22	16
PN007	DP	24	28	18	21	22	26	31	24	32	29	21	16
PN007	DP	27	0	28	35	0	0	24	23	35	0	22	0
PN009	DP	24	29	18	27	20	24	33	23	36	42	20	18
PN009	NBL	22	28	17	24	30	24	33	23	33	33	23	16
PN009	DP	23	29	16	22	30	18	32	22	31	28	22	16
PN015	DP	28	0	27	35	0	0	0	23	36	0	25	0
PN021	ETT	26	31	14	21	29	21	35	24	36	17	21	16
PN021	DP	27	29	17	20	30	24	36	23	39	16	19	16
PN021	NBL	28	30	18	20	30	25	35	22	36	15	23	16
PN021	DP	24	29	17	22	28	21	30	22	32	29	21	17
PN024	SUB	26	30	19	21	28	26	30	23	31	15	21	16
PN024	NBL	25	29	19	22	27	26	30	22	30	17	22	16
PN024	ETT	24	29	18	22	27	23	29	23	33	30	22	18
PN024	DP	23	30	19	26	30	23	31	22	30	39	20	18
PN024	NBL	27	28	16	28	29	22	32	23	33	36	21	19
PN025	DP	25	30	17	27	28	24	30	23	32	30	21	16
PN025	NBL	27	30	18	28	30	24	31	22	31	34	22	17

PN025	NBL	27	29	17	27	28	25	30	22	30	40	22	16
PN025	ETT	26	29	18	26	27	25	32	23	32	29	21	18
PN027	NBL	28	29	28	34	29	23	30	24	31	40	21	17
PN027	NBL	25	29	16	0	29	24	30	22	30	30	23	17
PN027	ETT	23	30	18	0	22	0	30	22	10	0	21	17
PN028	ETT	0	9	0	24	23	0	30	25	0	0	19	14
PN028	SUB	0	10	0	12	29	0	33	22	13	7	22	16
PN028	NBL	0	11	0	0	31	0	30	22	12	0	22	17
PN037	DP	24	30	18	26	28	18	31	25	30	17	20	18
PN037	NBL	25	30	19	27	24	27	30	23	30	17	21	17
PN037	NBL	31	30	20	25	28	26	30	24	30	19	19	18
PN037	NBL	24	30	17	24	28	22	30	24	30	20	20	20
PN042	DP	24	30	18	24	28	23	30	24	30	20	20	20
PN042	NBL	24	30	18	25	24	23	30	24	30	20	19	18
PN042	SUB	16	0	0	25	13	26	30	24	18	0	14	15
PN042	ETT	24	30	18	25	28	22	30	22	30	30	19	19
PN043	DP	24	30	18	24	28	22	30	24	30	18	18	18
PN045	DP	22	29	19	23	30	23	30	22	32	21	22	19
PN046	DP	24	30	18	25	28	22	30	22	30	28	19	19
PN046	NBL	24	30	18	25	28	22	30	22	30	28	19	19
PN046	DP	25	30	19	24	28	22	30	22	30	28	18	14
PN046	NBL	27	30	20	27	25	19	29	22	29	21	22	18
PN046	ETT	24	30	18	24	28	22	28	23	30	28	18	21
PN046	DP	22	30	20	23	30	22	29	0	31	18	20	18
PN048	NBL	25	30	18	24	24	18	30	22	30	28	20	20
PN049	DP	22	30	19	25	21	20	30	19	29	19	22	20
PN049	NBL	20	30	20	24	21	23	29	25	32	29	24	0

PN049	DP	25	0	18	11	10	22	19	20	30	20	14	0
PN050	DP	22	30	19	25	28	20	30	22	30	28	19	20
PN050	NBL	22	29	20	24	28	19	29	19	30	19	20	20
PN050	ETT	23	30	20	22	29	19	29	23	30	28	20	20
PN051	DP	23	30	19	0	28	0	29	19	30	28	24	20
PN051	DP	20	30	20	22	21	19	30	22	29	22	24	21
PN052	DP	24	31	18	24	0	0	29	22	30	28	22	19
PN052	NBL	23	30	19	24	30	23	29	22	30	29	21	4
PN052	ETT	21	30	19	22	21	22	29	22	30	20	20	11
PN054	NBL	23	30	20	24	22	23	30	22	30	21	19	19
PN056	DP	23	30	20	22	29	19	29	22	30	19	22	19
PN056	NBL	23	30	20	22	29	19	30	24	30	29	19	21
PN056	DP	19	29	21	22	22	23	30	18	30	29	21	19
PN056	NBL	20	30	19	22	29	23	30	24	29	28	21	19
PN056	SUB	19	30	21	22	21	23	30	18	30	28	22	20
PN056	SUB	19	30	21	23	21	22	30	18	28	21	21	21
PN057	NBL	19	29	20	23	22	22	30	22	30	19	21	22
PN057	ETT	20	30	18	23	22	19	29	23	30	21	21	21
PN058	SUB	19	29	20	21	22	22	29	18	30	30	22	0
PN063	NBL	20	29	18	22	22	19	29	18	30	30	22	0
PN068	DP	21	30	20	0	9	0	30	20	30	30	20	0
PN068	NBL	22	30	20	22	28	0	26	21	29	28	0	28
PN068	DP	22	30	19	21	28	28	11	21	30	29	21	19
PN077	NBL	22	29	19	21	27	22	29	20	21	22	19	19
PN077	DP	22	30	19	26	28	22	30	20	32	23	20	0
PN077	NBL	21	30	20	22	28	22	30	20	30	29	21	19
PN080	SUB	0	14	0	10	28	0	30	10	20	0	10	21

PN080	DP	0	13	0	0	32	0	30	10	21	0	10	21
PN080	DP	0	15	0	0	33	0	33	11	21	0	0	21
PN081	NBL	0	0	0	0	28	23	10	27	30	0	20	0
PN081	SUB	0	0	0	0	32	26	29	23	7	0	20	9
PN081	DP	0	9	0	0	33	29	31	24	14	0	23	18
PN081	DP	0	8	0	0	30	27	21	24	9	0	22	17
PN086	NBL	23	30	19	23	28	22	29	22	29	21	22	20
PN087	ETT	21	30	19	23	28	19	30	24	29	21	24	20
PN087	NBL	25	20	16	21	29	8	29	22	29	17	23	20
PN087	SUB	24	30	19	24	22	0	30	22	30	28	22	20
PN087	DP	23	30	20	22	28	0	30	22	29	29	21	20
PN089	NBL	23	30	20	24	28	22	30	24	30	29	21	19
PN089	SUB	23	29	21	22	21	22	29	22	30	28	20	21
PN089	NBL	25	30	22	23	28	24	29	23	29	31	20	20
PN089	DP	21	30	21	24	28	22	29	23	29	28	21	19
PN092	DP	23	30	22	0	21	23	30	22	27	24	21	20
PN092	NBL	21	30	20	23	21	24	29	23	29	29	22	20
PN094	NBL	24	29	21	22	28	23	30	22	29	29	21	19
PN095	DP	21	29	21	22	0	0	29	22	29	32	21	20
PN095	NBL	29	32	17	22	0	0	17	25	35	29	22	19
PN095	SUB	26	30	14	24	0	0	19	24	33	29	18	18
PN095	DP	22	30	18	24	0	0	19	23	29	18	21	0
PN097	NBL	23	30	21	24	24	23	30	21	31	31	22	18
PN099	DP	20	29	23	24	28	22	30	23	30	28	21	21
PN099	DP	23	30	19	22	27	19	29	23	30	29	21	18
PN102	DP	23	30	19	23	25	22	29	22	33	29	19	19
PN102	DP	20	29	22	21	0	0	30	22	34	30	21	20

PN104	DP	21	30	24	23	28	24	30	24	30	21	21	17
PN104	NBL	21	30	21	23	21	25	30	24	30	29	22	18
PN104	DP	20	30	22	24	21	23	30	23	30	28	20	21
PN105	NBL	20	30	21	20	28	23	30	23	30	31	22	19
PN105	ETT	24	30	22	21	21	24	30	23	29	29	20	18
PN106	ETT	22	30	23	21	28	23	29	23	30	30	21	19
PN108	DP	20	30	22	23	21	23	30	23	30	28	19	20
PN108	NBL	24	30	19	23	28	22	29	22	29	28	21	20
PN108	ETT	21	30	16	23	29	22	31	24	30	28	20	19
PN109	DP	20	30	17	24	24	22	32	22	32	30	20	17
PN110	NBL	0	0	0	10	32	0	25	12	0	0	12	20
												Resistant	

Supplementary table 1 – Individual antimicrobial sensitivities for *S. aureus* isolates obtained during mechanical, and where possible, into the post- endotracheal tube extubation recovery period.

Patient		Antibiotics						
Patient	**Sample**	**Ceftazidime**	**Ciprofloxacin**	**Gentamicin**	**Meropenem**	**Piperacillin**	**Piperacillin-Tazobactam**	**Tobramycin**
PN006	DP	22	30	20	27	24	27	19
PN007	DP	22	30	20	28	24	27	22
PN008	DP	24	30	26	28	24	27	21
PN018	DP	22	30	19	27	24	28	21
PN018	NBL	23	30	18	32	25	27	20
PN030	DP	19	30	19	28	23	27	20
PN030	NBL	20	30	19	29	24	27	20
PN030	SUB	21	29	20	30	24	27	19
PN030	NBL	22	29	20	30	24	29	19

PN030	ETT	23	30	18	32	20	26	20
PN030	DP	19	31	19	31	18	26	20
PN038	DP	22	30	24	0	18	20	20
PN045	NBL	22	30	22	0	17	21	22
PN047	DP	22	29	19	20	21	26	24
PN047	DP	19	28	21	27	22	29	22
PN047	DP	22	30	19	27	23	26	19
PN047	DP	21	30	20	27	19	27	20
PN048	NBL	22	30	20	28	23	27	21
PN049	DP	21	30	19	28	17	18	19
PN049	DP	22	30	18	18	23	26	21
PN051	DP	21	30	20	27	23	28	21
PN060	DP	23	30	21	27	21	25	22
PN060	DP	22	30	21	18	14	19	21
PN060	DP	21	30	19	29	21	28	19
PN062	DP	22	31	21	29	22	27	19
PN062	NBL	22	28	21	28	22	27	19
PN062	DP	22	30	20	0	23	25	20
PN075	DP	20	30	20	29	21	26	19
PN078	NBL	21	30	20	28	20	28	20
PN079	SUB	22	30	18	30	21	26	20
PN079	DP	22	30	21	28	0	14	19
PN079	NBL	23	30	21	29	19	28	22
PN079	DP	22	27	19	6	11	15	21
PN079	DP	22	27	19	0	12	17	21
PN081	DP	23	30	20	0	21	26	20
PN083	NBL	23	30	21	10	19	27	21

PN095	SUB	22	29	20	29	20	29	19
PN095	DP	20	29	21	29	21	28	22
PN095	ETT	22	29	21	29	19	29	19
PN095	DP	22	30	21	0	18	29	18
PN095	NBL	21	30	18	10	22	29	19
PN095	SUB	21	30	23	28	23	27	20
PN102	DP	21	30	20	30	24	28	20
PN104	DP	20	30	19	30	21	28	20
PN105	ETT	24	30	19	28	24	27	21
PN105	NBL	25	30	19	29	24	29	21
PN105	DP	21	29	21	29	21	28	21
PN106	DP	19	29	21	27	23	27	19
PN107	DP	22	30	21	30	22	28	20
PN108	ETT	16	7	17	19	11	19	14
PN109	DP	20	30	21	31	23	28	20
PN109	DP	23	30	21	27	23	29	20
PN109	DP	22	29	21	29	25	28	19
PN110	DP	22	28	20	28	21	25	20
PN110	NBL	23	27	21	28	22	26	19
PN110	ETT	22	30	21	29	22	26	21
							Resistant	

Supplementary table 2 - Individual antimicrobial sensitivities for *P. aeruginosa* isolates obtained during mechanical, and where possible, into the post- endotracheal tube extubation recovery period.