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Endotracheal tube biofilm inoculation of oral flora and subsequent colonization of opportunistic pathogens

Sarah D. Perkins^a, Keith F. Woeltje^b, Largus T. Angenent^{c,*}

^a Department of Energy, Environmental & Chemical Engineering, Washington University in St. Louis, St. Louis, MO 63130, USA

^b Division of Infectious Disease, Washington University in St. Louis School of Medicine, St. Louis, MO 63108, USA

^c Department of Biological and Environmental Engineering, Cornell University, Ithaca, NY 14853, USA

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ABSTRACT

Endotracheal (ET) tubes accumulate a biofilm during use, which can harbor potentially pathogenic microorganisms. The enrichment of pathogenic strains in the biofilm may lead to ventilator-associated pneumonia (VAP) with an increased morbidity rate in intensive care units. We used quantitative PCR (qPCR) and gene surveys targeting 16S rRNA genes to quantify and identify the bacterial community to detect fastidious/nonculturable organisms present among extubated ET tubes. We collected eight ET tubes with intubation periods between 12 h and 23 d from different patients in a surgical and a medical intensive care unit. Our qPCR data showed that ET tubes were colonized within 24 h. However, the variation between patients was too high to find a positive correlation between the bacterial load and intubation period. We obtained 1263 near full-length 16S rRNA gene sequences from the diverse bacterial communities. Over 70% of these sequences were associated with genera of typical oral flora, while only 6% were associated with gastrointestinal flora. The most common genus identified was *Streptococcus* (348/1263), followed by *Prevotella* (179/1263), and *Neisseria* (143/1263) with the highest relative concentrations for ET tubes with short intubation periods, indicating oral inoculation of the ET tubes. Our study also shows that even though potentially pathogenic bacteria existed in ET tube biofilms within 24 h of intubation, a longer intubation period increases the opportunity for these organisms to proliferate. In the ET tube that was in place for 23 d, 95% of the sequences belonged to *Pseudomonas aeruginosa*, which is a bacterial pathogen that is known to out compete commensal bacteria in biofilms, especially during periods of antibiotic treatment. Harboring such pathogens in ET biofilms may increase the chance of VAP, and should be aggressively monitored and prevented.

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Introduction

Mechanical ventilation is a life-saving medical strategy for trauma and medically incapacitated patients; however, it is not free of risk. Ventilator-associated pneumonia (VAP) is one of the most common acquired infections in intensive care units (ICUs) (Agbaht et al., 2007; Mayhall, 2007; Rello et al., 2006). The CDC (2005) reports that patients who are being mechanically ventilated have a 6–21 times higher risk of developing hospital-associated pneumonia compared with patients who are not receiving this treatment. With increasing public scrutiny of healthcare-associated infections, hospitals are working to implement prevention strategies to minimize the risk of developing VAP (Coffin et al., 2008).

A variety of bacteria have been closely associated with VAP, including *Staphylococcus* spp., *Pseudomonas* spp., and *Acinetobacter* spp. (Agbaht et al., 2007; Masterton et al., 2007; Schultz and Spronk,

2007). In some diagnosed VAP cases, the offending organism was not identifiable through standard laboratory culturing techniques (Bahrani-Mougeot et al., 2007; Kollef et al., 2005; Weber et al., 2007). A number of prevention studies have attempted to reduce or eliminate the risk of VAP by looking into the effects of oral decontamination, ventilator tube changes, patient positioning, antibiotic therapies, continued education, silver-coated tubes, and combinations of the aforementioned (Blot et al., 2007; Coffin et al., 2008; Garnacho-Montero et al., 2007; Gastmeier and Geffers, 2007; Kollef et al., 2008; Omrane et al., 2007).

Endotracheal (ET) tubes are placed through the highly colonized oropharynx and larynx into the normally sterile tracheobronchial tree, creating a direct passage from an external ventilator to the patient's lungs (Safdar et al., 2005). Biofilms, which are complex communities of microbes that produce glycocalyx polysaccharides to protect them from desiccation, chemical treatments, and immunological attack (Edwards, 2000), have been found along the inner and outer surfaces of ET tubes after being in place for less than 24 h (Augustyn, 2007; Inglis et al., 1989; Ramirez et al., 2007; Sottile et al., 1986). Hypotheses on the origins of the biofilm have

* Corresponding author. Tel.: +1 607 255 2480; fax: +1 607 255 4449.

E-mail address: la249@cornell.edu (L.T. Angenent).

ranged from the progressive accumulation of an individual's lower respiratory tract secretions during inadequate suctioning (Inglis et al., 1995), colonization of aspirated secretions from the upper respiratory tract (Bahrani-Mougeot et al., 2007), and environmental inoculation of forced air from ventilators (Inglis et al., 1989). Regardless of the inoculum source, researchers are confident that biofilms are a reservoir of potentially pathogenic bacteria that could propagate into systemic infections (Di Filippo and De Gaudio, 2003; Pacheco-Fowler et al., 2004; Ramirez et al., 2007).

Most previous studies have focused on linking tube biofilm culture analysis with cultured specimens from the patient and visualizing collected biofilms with scanning electron microscopy as a function of time and location (Adair et al., 1999; Inglis et al., 1989, 1995; Sottile et al., 1986; Zur et al., 2004). However, direct links between biofilm formation and VAP incidences have not been definitively observed. Each of these studies relied on culturing techniques to determine what organisms were present, yet studies have shown that there can be significant culturing bias (Amann et al., 1995; Edwards, 2000; Pace, 1997). To circumvent this culturing bias, molecular techniques have been developed to characterize microorganisms without a culturing step. During gene surveys to identify bacteria, DNA is directly extracted from the specimen and the bacterial small-subunit rRNA (16S rRNA) gene is amplified by PCR, sequenced, and identified through a phylogenetic approach (Kroes et al., 1999; Ley et al., 2006; Paster et al., 2001). Notably, the 16S rRNA gene has withstood horizontal gene transfer, making it highly conserved and an ideal tool for identifying microorganisms that are nonculturable or nonviable (Woese, 1987). Bahrani-Mougeot et al. (2007) and Flanagan et al. (2007) have each assessed intubated patients with nonculturing techniques. Bahrani-Mougeot et al. (2007) sought to correlate bacterial pathogens from bronchoalveolar lavage samples to oral swabs collected from patients with clinically diagnosed VAP. Alternatively, Flanagan et al. (2007) used endotracheal aspirates to determine the overall change in phylogenetic diversity with antibiotic use in intubated patients cultured with *Pseudomonas* spp.

To our knowledge, all studies with nonculturing techniques have sampled tracheal aspirates or bronchoalveolar lavage fluids rather than ET tube biofilms, which is advantageous for a time-resolved sampling study because ET tubes are not removed during ventilation to prevent infection. However, such studies will not completely ascertain the pool of pathogens present because biofilms harbor specific pathogens. Here, we sampled the entire biofilm surrounding eight extubated ET tubes from different patients. These eight ET tubes had been in place for conventional intubation periods between 12 h and 23 d to mimic time-resolved sampling. We performed 16S rRNA gene surveys on biofilm communities from a relatively large tube surface area. The sequencing was performed with a Sanger-sequencing platform to generate near full-length sequencing reads to identify pathogens on a species level. We also used a direct quantitative PCR (qPCR) approach to quantify the bacterial loads. The information from these nonculture-based techniques allowed us to identify potential pathogens in the biofilm, and gave us the opportunity to postulate the period during which these pathogens proliferated, the likely inoculum source, and possible interventions to further reduce the incidence of VAP.

Materials and methods

Patient recruitment and sampling

The study population consisted of patients selected on a basis of ventilation duration from the Barnes-Jewish Hospital Medical Intensive Care Unit (ICU) and Surgical ICU. The patients were all

above the age of 17 with mechanical ventilation for 12 h to 23 d (mean 6 d). Barnes-Jewish Hospital is a 1252-bed tertiary care teaching hospital affiliated with Washington University School of Medicine, St. Louis, MO. The institutional review board of Barnes-Jewish Hospital approved a verbal consent to be obtained from either the patient or family following physician-prescribed extubation allowing study enrollment and access to patient information. Results of cultures obtained for clinical reasons and antibiotic treatment were acquired from the patients' charts retrospectively (Table 1). The extubated tubes were deposited in sterile bags and kept on ice (less than 12 h) until ET tube duration was determined. Not all tubes were further studied. We chose eight ET tubes for further study based on the length of intubation period. The samples are classified as A–H from the shortest to the longest intubation period (Table 1).

Sample preparation and DNA extraction

Each tube was segmented into three equal portions (~10 cm each) from the oral cavity down: proximal, medial, and distal. The ET tube pieces were individually stored at -80°C until DNA extraction. A rigorous extraction protocol adapted from Cury and Koo (2007) was used to maximize the DNA yield from within the accumulated biofilm encasing the interior and exterior of the removed ET tubes. In short, the ET tube segments were removed from the -80°C freezer and further cut down to 0.5-cm long tubes, which were cut longitudinal into four pieces. A 25-ml sterile phosphate buffer solution (PBS) was added to the pieces and stored at -80°C overnight. The samples were thawed and subjected to three rounds of 30-s vortex, 60-s sonication at 7 W, and 10-min rest in an ice bath. The PBS was transferred into a new vial and the ET tube pieces were washed with two additional 10-ml aliquots of PBS. The PBS effluent from each ET segment was centrifuged at 5,040 g for 10 min (Sorvall RC-5B, Thermo Fisher Scientific, Waltham, MA) and decanted. The remaining cell pellet was resuspended in extraction buffer and subjected to a bead-beating phenol–chloroform extraction protocol (Ley et al., 2006).

DNA quantification

Quantitative PCR (qPCR) was used to determine the bacterial DNA load within each sample in triplicate using SYBR green chemistry. Wells contained 2 μl of extracted template and 23 μl of SYBR green mix (ABgene, Rockford, IL) supplemented with 0.25 U UDP-N-glycosidase, and 10 μM universal bacterial primers (forward primer 331F 5'-TCCTACGGGAGGCAGCAGT-3'; reverse primer 797R 5'-GGACTACCAGGTATCTAATCCTGTT-3'; Integrated DNA Technologies, Coralville, IA) to target 16S rRNA genes (Nadkarni et al., 2002). Triplicate samples were analyzed with a Stratagene Mx3000P qPCR system (Cedar Creek, TX), using the program outlined and verified by An et al. (2006) (i.e., 40 cycles at 95°C for 15 s, 1 min at 60°C , 30 s at 72°C with data collection temperatures of 85°C to 88°C and analysis of a final melting curve). To generate a standard curve for qPCR, DNA was extracted from *Escherichia coli*, and quantified using a PicoGreen dsDNA Quantitation Kit (Molecular Probes, Eugene, Oregon). The standard curve of *E. coli* DNA ranged from 0.034 pg/l to 34.7 ng/l with an R^2 of 0.94. An analysis of variances (ANOVA) test was performed with the qPCR data using R Project for statistical computing software (www.r-project.org). A two-way ANOVA was used to determine the significance of patient and ET tube section location. A correlation analysis was also conducted to determine the correlation between the concentration of DNA along each ET tube section and the duration of intubation. For this analysis it was assumed that each data set had a bivariate normal distribution.

Table 1
Individual ET tube details.

ET tube	Intubation duration	Laboratory results	Non-chimeric sequences	OTUs	RDP % match to potential pathogen	Matching sequences	Potential pathogen	Antibiotic treatment
A	12 h	No positive cultures	171	61	98.2	1	<i>Fusobacterium nucleatum</i> ; DQ440557	None
					97.6	2	<i>Granulicatella adiacens</i> ; AY879300	
B	18 h	Day 1: MRSA from a nasal swab PCR	193	47	99.1	3	<i>Abiotrophia para-adiacens</i> ; AB022027	None
					99.3	61	<i>Neisseria perflava</i> ; AJ247246	
C	23 h	No positive cultures	151	56	97.2	1	<i>Haemophilus parainfluenzae</i> ; AY362908	Cefoxitin
					97.7	4	<i>Neisseria perflava</i> ; AJ247246	
D	2 d	Day 1: MRSA from a trachea aspirate	87	36	98.2	2	<i>Abiotrophia para-adiacens</i> ; AB022027	Cefepime, linezolid
					98.0	1	<i>Staphylococcus aureus</i> ; BA000017	
E	6 d	Day 1: MRSA from a nasal swab PCR	102	30	97.2	1	<i>Haemophilus parainfluenzae</i> ; AB098612	Ceftriaxone, ampicillin, metronidazole, vancomycin
					99.1	7	<i>Abiotrophia para-adiacens</i> ; AB022027	
					100	2	<i>Neisseria perflava</i> ; AJ247246	
F	7 d	No positive cultures	223	62	97.8	20	<i>Neisseria mucosa</i> ; AJ239279	Cefazolin, gentamicin
G	7 d	Day 3: <i>Enterococcus</i> spp. in the urine	212	83	98.5	1	<i>Fusobacterium nucleatum</i> ; DQ440565	Ciprofloxacin, ampicillin
					97.8	2	<i>Enterococcus faecalis</i> ; AB272354	
					97.1	1	<i>Prevotella nigrescens</i> ; AF414842	
H	23 d	Day 2: MRSA from a nasal swab PCR	124	23	100	2	<i>Staphylococcus aureus</i> ; CP000046	Ceftriaxone, ciprofloxacin, metronidazole, cefepime, vancomycin, linezolid, meropenem
		Day 18: <i>Pseudomonas aeruginosa</i> from a trachea aspirate			100	117	<i>Pseudomonas aeruginosa</i> ; AY826613	
Total			1263	331 ^a		228		

^a Total number of independent OTUs for the study.

PCR amplification, cloning, and sequence analysis

16S rRNA genes from extracts of each section of the ET tubes were PCR amplified with a 30-cycle touchdown PCR (initial 2 min denaturing step at 94 °C, followed by 30 cycles at 92 °C for 30 s, 45 °C for 90 s, and 72 °C for 90 s, and a final 72 °C extension for 15 min). The 50-µl reaction mixes contained 1.25 U of GoTaq (Promega Corp, Madison, WI), 0.4 pmol/µl forward and reverse universal primers (8F 5'-AGAGTTTGATCCTGGCTCAG-3'; 1391R 5'GACGGGCGGTGWGTRCA-3'; Integrated DNA Technologies) targeting bacterial 16S rRNA genes (Lane et al., 1985), 0.5 mM MgCl₂, 0.2 mM dNTPs, 0.8 mg/ml BSA, and 2 µl of template. Positive and negative control reactions were included with each reaction set, and BSA was added to impede amplification inhibition. PCR-amplified products of the proximal, medial, and distal portions of the ET tubes were combined, cleaned, and concentrated (Montage PCR cleaning kit, UFC7 PCR50, Millipore, Billerica, MA) before being sent to the Genome Sequencing Center, Washington University School of Medicine, for cloning, purification, and Sanger sequencing on an ABI 3730 capillary sequencer (Applied Biosys-

tems, Foster City, CA). 16S rRNA gene sequences were edited and assembled into consensus sequences using PHRED and PHRAP aided by XplorSeq (Frank, 2008). Bases with a PHRAP quality score <20 were trimmed from the dataset and chimeras were detected and removed using Bellerophon (Huber et al., 2004). Next, the remaining sequences were aligned using the NAST online tool (DeSantis et al., 2006a) to determine the rRNA secondary structure information with phylogenetic identification. These sequences were then compared to the greengenes public database and the alignment of each sequence with the three closest matches were imported into the computer application software ARB (Ludwig et al., 2004). A pair-wise identity of 97% or greater was used to bin sequences into "species"-level operational taxonomic units (OTUs) (Goebel and Stackebrandt, 1994; Stackebrandt and Ebers, 2006). The generated distance matrices were used in the program DOTUR to assess the number of OTUs and to cluster them by pair-wise identity with a furthest-neighbor algorithm (Schloss and Handelsman, 2005). Assignment of the majority of sequences to phyla was based on their position after parsimony insertion to the ARB dendrogram in the greengenes database (DeSantis et al., 2006b). Phyla

classifications were double-checked and sequence match scores (%ID) were calculated for non-chimeric sequences with the Ribosomal Database Project II (RDP) (Cole et al., 2007). The program jModelTest 0.1 with an Aikai Information Criterion to rank 88 candidate models with an objective scoring method (Posada, 2008) was used to identify the best phylogenetic tree model. The best-fit model was then analyzed through a heuristic algorithm with PAUP (Wilgenbusch and Swofford, 2003). Finally, UniFrac was used to conduct a jackknife replication weighted test, replicated 100 times to compare the eight ET tube biofilm environments by clustering them based upon the diversity and relative abundance of the sequences found within each sample (Lozupone et al., 2006).

Nucleotide sequence accession numbers

Near full-length 16S rRNA gene sequences were deposited in GenBank under accession numbers FJ557249–FJ558511.

Results

Bacterial quantification

We measured the relative concentration of 16S rRNA genes (measured as ng of bacterial DNA) from three segments for eight ET tubes with qPCR to report a log average DNA load per 9-cm long segment of ET tube (Fig. 1). Within 24 h of intubation in the patient, a bacterial biofilm had been formed on the proximal, medial, and distal sections of ET tubes B and C. Even after a 12-h intubation period, a relatively large quantity of bacterial DNA was observed on the proximal section of the ET tube A (Fig. 1). We found a statistically significant correlation of the DNA load for proximal, medial,

and distal sections of each ET tube within patients ($p < 0.001$), but not between patients ($p = 0.06$). In addition, a multiple correlation analysis between the duration of intubation and biofilm DNA load for each segment (proximal, medial, or distal) was not statistically significant ($p = 0.17$). Thus, from this work we cannot conclude whether bacterial loads in biofilms increase over the intubation period due to the large variation in DNA loads.

Sequence analysis

Directly collected DNA extracts from the uncultured ET tubes were pooled from the proximal, medial, and distal sections, and characterized with a gene sequencing survey. A total of 1263 non-chimeric, near full-length 16S rRNA gene sequences were generated in this study. Distance-based operational taxonomic unit (OTU) analysis resulted in 331 different OTUs based on a 1% cut-off (Table 1). The phylogenetic distribution of all the sequences collected in this study exemplifies the diversity of abundant microbes found in these biofilms. When all 1263 sequences from ET tube biofilms were pooled, they represented five different phyla: *Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, *Fusobacteria*, and *Firmicutes* (Fig. 2). For the individual ET tubes, sequences from: (1) the phyla *Actinobacteria* and *Firmicutes* were found in each of the ET tube biofilms even though there was a considerable variance in their relative concentrations; (2) the phyla *Proteobacteria* and *Bacteroidetes* were found within all the biofilms except for ET tube G and ET tube H; and (3) the phylum *Fusobacteria* was only present in biofilms of ET tubes A, B, and C, which were in place for 12 h, 18 h, and 24 h, respectively (Fig. 2). This phylum was identified at relatively low levels and its abundance decreased from 1.8%, 1.0%, and 0.6% with an increasing intubation period for ET tubes A, B, and C, respectively (Fig. 2).

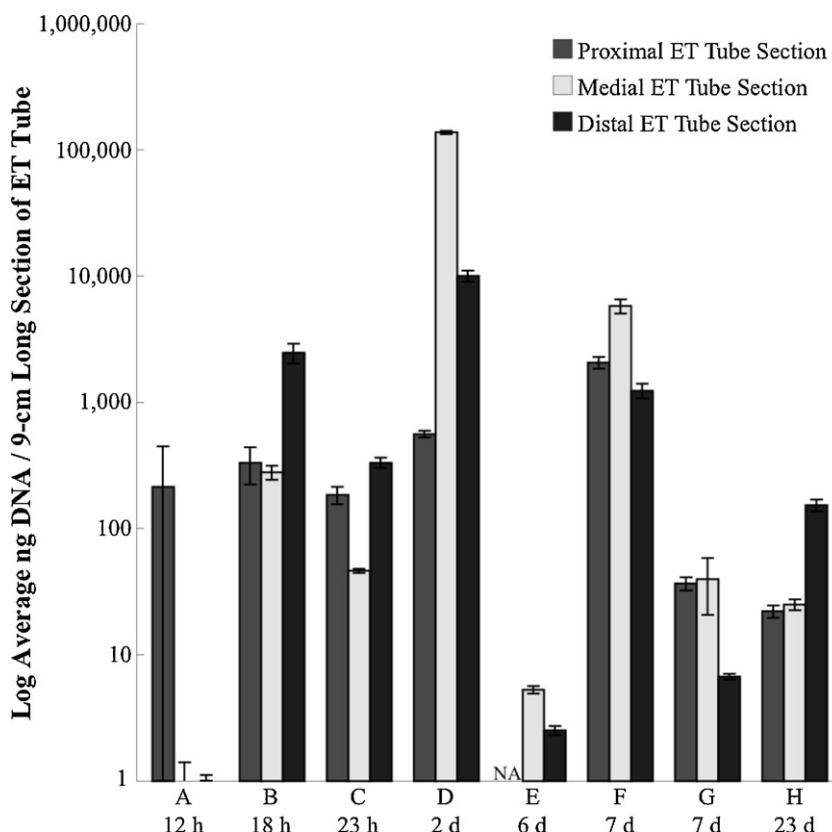


Fig. 1. Quantitative PCR log average concentration of bacterial 16S rRNA gene DNA found within the biofilm of triplicate samples of each 9-cm long ET tube section. Error bars indicate the 95% confidence intervals of triplicate qPCR tests for each ET tube section.

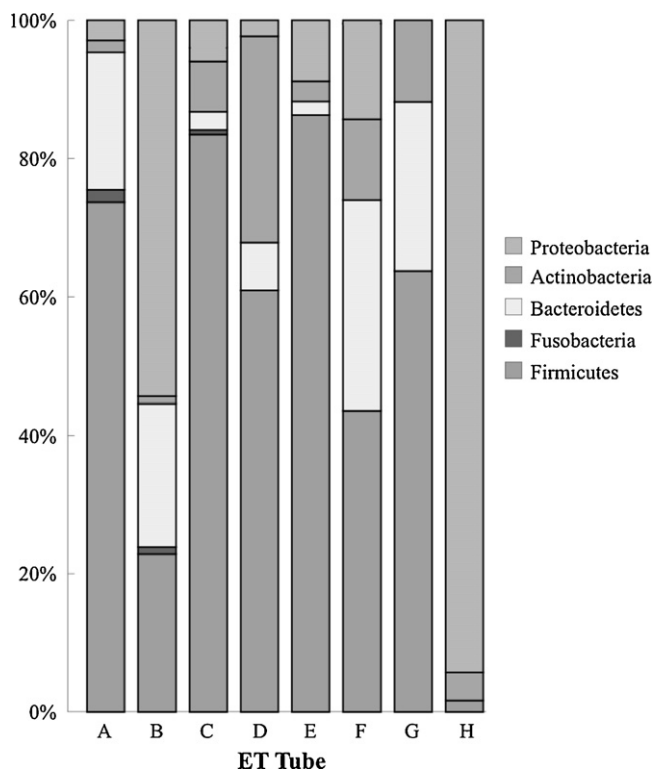


Fig. 2. Phylum comparison of sequences from each ET tube sample.

The RDP sequence match algorithm was used to identify the sequences from ET tube biofilms with a specific interest to identify potential pathogenic bacteria. Out of 1263 non-chimeric sequences, 228 were >97% identical to known potential pathogens (Table 1). The most notable potential pathogens identified included *Pseudomonas aeruginosa*, *Haemophilus parainfluenzae*, *Staphylococcus aureus*, *Fusobacterium nucleatum*, *Neisseria perflava*, and *Prevotella nigrescens* (Table 1). To further verify the relatedness of these potential pathogens to cultured and uncultured sequences in public databases, we performed maximum likelihood, maximum parsimony, and neighbor joining analyses on the sequences that were identified within four bacterial phyla: (1) *Proteobacteria*, *Fusobacteria*, and *Bacteroidetes* (Fig. 3A); and (2) *Firmicutes* (Fig. 3B). These analyses produced similar trees, with similar bootstrap support at resolved branches (data not shown), and here we show maximum likelihood trees. Several sequences from our study were closely related to multiple uncultured oral sequences within the phyla of *Proteobacteria*, *Fusobacteria*, and *Bacteroidetes* from public databases (besides being related to cultured sequences), identifying some overlap of oral flora to ET tube biofilm flora. For the phylum *Proteobacteria*, 117 sequences in 21 different OTUs from ET tube H were found to be similar to *P. aeruginosa* (Fig. 3A). Four ET tubes (B, C, E, and F) exhibited members of the genus *Neisseria* in 20 different OTUs. We found 49 different OTUs that were similar to the genus *Prevotella* and that were diverse (Fig. 3A). Similarly, 74 OTUs with sequences from different ET tubes (except tube H) were dispersed within *Streptococcus* spp. (Fig. 3B). In addition, some of these OTUs were similar to uncultured oral and fecal clones (Fig. 3B). Two sequences (in different OTUs) from ET tube G were similar to sequences of the potentially pathogenic *Enterococcus faecalis*. Likewise, two sequences from ET tube H were closely related to the potentially pathogenic *S. aureus* (Fig. 3B). Sequences from multiple ET tubes were found to belong to a single OTU (i.e., in the genera *Neisseria*, *Haemophilus*, *Fusobacterium*, *Prevotella*, *Streptococcus*, *Abiotrophia*, and *Staphylococcus*), indicating that different patients carried very similar microbes.

A weighted environmental clustering test differentiated the bacterial communities in the ET tubes (Fig. 4). The biofilm community from ET tubes B and F and from A, C, D, E, and G were clustered. Among all biofilm communities, the bacterial composition for tube A and C were most similar to each other (with relatively short intubation periods), while ET tube H (with the longest intubation period) was characterized with a bacterial community that was most different from all other tubes. The phylum *Proteobacteria* played an important role in the weighted differentiation of the tube communities because of the presence/absence of very abundant proteobacterial species in the biofilm. More than 95% of the sequences from ET tube H were similar to *Pseudomonas* spp. sequences while they were not found in any of the other ET tubes, explaining the differentiation of tube H. In addition, 52% and 14% of the sequences in ET tubes B and F, respectively, belonged to *Neisseria* spp., partly explaining why the communities for these tubes clustered together.

Discussion

Our study clearly identified potential pathogens that are harbored in the biofilms of ET tubes. To obtain this information, we extubated ET tubes and physically removed the biofilms to be included in the analysis. Here, we discuss additional findings from this work. We realize that the patient population for this study was heterogeneous and small ($n = 8$), and therefore further studies with a larger patient population are necessary to investigate if the trends that we have postulated from this heterogeneous group remain valid.

Significant spatial differences in bacterial loads on ET tubes were not found

Studies have attempted to use fractal dimensions, porosity, and weight to quantify biofilms (Yang et al., 2000). This study used qPCR with universal bacterial primers to estimate the relative quantity of bacterial DNA (An et al., 2006; Nadkarni et al., 2002), which accumulated along three segments of eight collected ET tubes. Herein the standard curve was based upon a pure *E. coli* culture, but within the actual samples we have shown that there is indeed a large variation of bacterial species present that may have different 16S rRNA operon copy numbers. For future work a more accurate standard curve may be from a mix of oral and gastrointestinal (GI) bacterial species found within this study. Multiple reports have previously found a higher biofilm presence in the distal third of ET tubes (Adair et al., 1999; Feldman et al., 1999; Koerner, 1997; Zur et al., 2004) due to build up of respiratory secretions. However, here we did not find that this correlated with a higher bacterial load in the distal third of ET tubes compared to the proximal and medial thirds (Fig. 1). In addition, a clinically observed tendency for mucus accumulation on ET tubes over time (Adair et al., 1999; Feldman et al., 1999; Koerner, 1997; Zur et al., 2004) did not necessarily correlate with biofilm or quantitative culture analyses (Zur et al., 2004; Inglis et al., 1989). We also did not determine an accumulation of bacteria in the biofilm over the intubation period.

Oral communities likely inoculated the ET tubes

The most common genus identified in this study was *Streptococcus* (348/1263). The presence of *Streptococcus* species in all but the ET tube H biofilm (Fig. 3) was not surprising because *Streptococcus* spp. are considered normal flora of the nasopharynx and upper respiratory tract through which ET tubes are placed (Wolf and Daley, 2007). However, *Streptococcus* spp. has the ability to coaggregate with other microbes and form biofilms. This gives them the ability to start colonization and initiate the formation of biofilms (Cury and

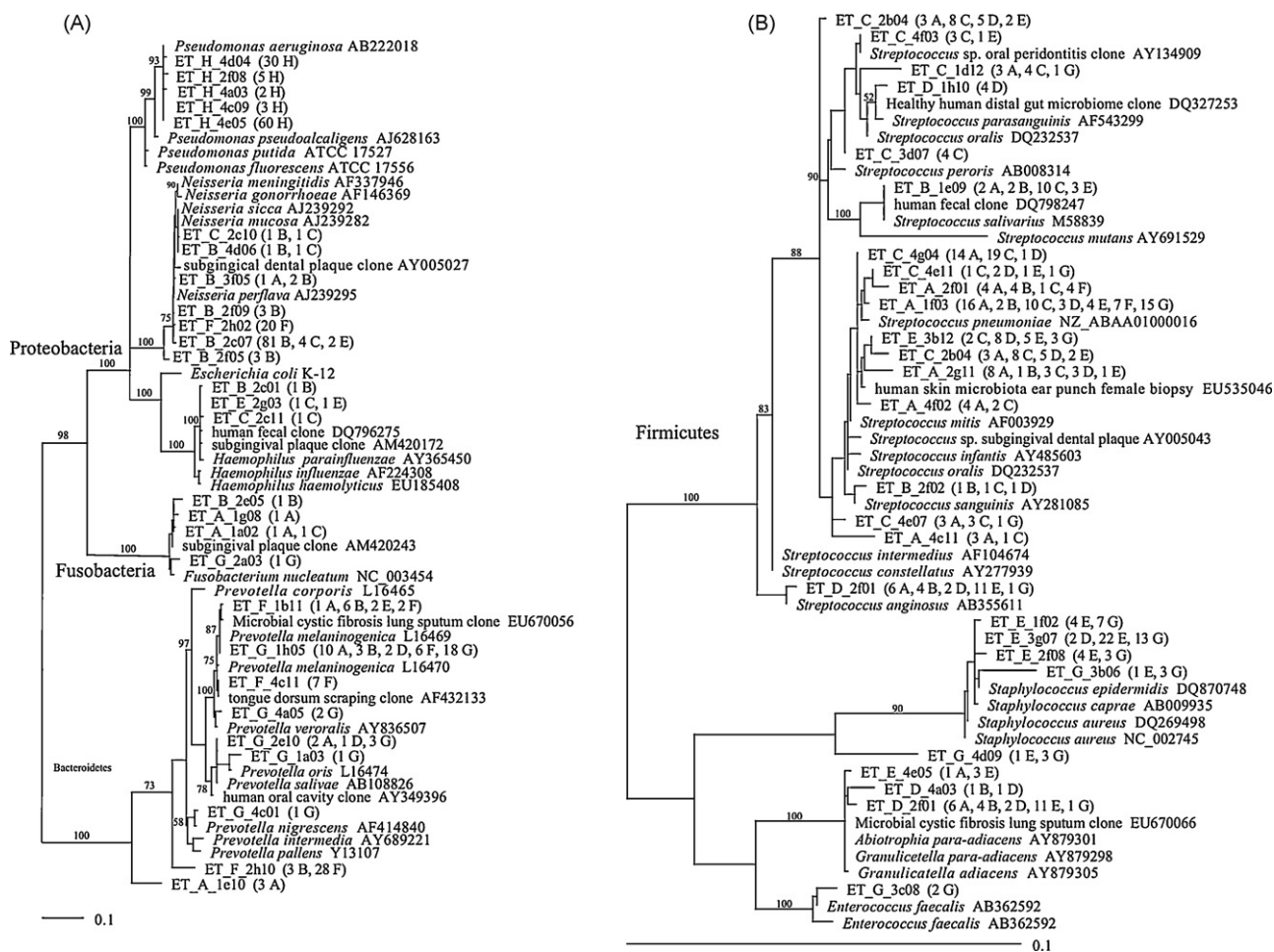


Fig. 3. Maximum likelihood phylogenetic distribution of select 16S rRNA gene sequences collected in this study from the phyla (A) *Proteobacteria*, *Fusobacteria*, and *Bacteroidetes* and (B) *Firmicutes*. Reference sequences from the NCBI database are included with their accession numbers. Individual sequences that represent one OTU are labeled with “ET” followed by the sample label and the unique sequence identity. The number of sequences in the OTU found in this study is given in parentheses following the sequence identity. The scale bar represents base changes per site. Only the OTUs with a large similarity to cultured sequence entries are shown. The bootstrap numbers are based on the neighbor-joining method.

Koo, 2007; Diaz et al., 2006). Specific cystic metabolic relationships have been established within oral microbial flora between *Streptococcus* spp. and *Actinomyces* spp., and *Streptococcus* spp. and *Veillonella* spp. (Diaz et al., 2006; Palmer et al., 2003). *Streptococcus* spp. and

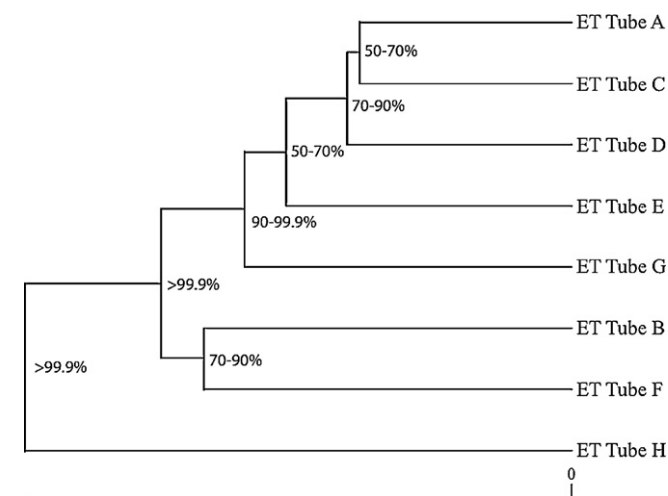


Fig. 4. Environmental cluster analysis to differentiate each ET tube biofilm environment. The percentage of times each node was recovered in the 100 replicate analyses is indicated.

Actinomyces spp. were both found within all ET tube biofilms except for ET tube H, and *Streptococcus* spp. and *Veillonella* spp. were found in ET tube biofilms A, B, C, E, and F. In addition, these two pairs of associations were found in all ET tube biofilms that were in place for less than 24 h, verifying that they may be important for biofilm formation. Over 70% of the sequences (894/1263) that were collected from these ET tubes were associated with genera of typical oral flora. This verifies previous findings that superior concentrations of natural oral flora are responsible for the development of biofilm that can ultimately cause VAP (Bahrani-Mougeot et al., 2007; Jones and Munro, 2008). Bahrani-Mougeot et al. (2007), in particular, found that 88% of the patients in their study diagnosed with VAP had overlapping pathogens within the bronchoalveolar lavage fluid and oral cavity. Only 6% (74/1263) of sequences found in our study were among genera of typical GI flora. Our work, thus, supports the findings of Garrouste-Orgeas et al. (1997), who determined that GI colonization was not the primary source of pathogens responsible for VAP even though GI flora can colonize ET tubes with refluxed gastric fluids.

Potentially pathogenic bacteria were able to colonize ET tube biofilms

Approximately 20% of the sequences collected in this study were similar to sequences from known potentially pathogenic organ-

isms (Table 1). Further analyses with phylogenetic tools, such as with the maximum likelihood algorithm, confirmed these results (Fig. 3). Six of the potential pathogens that were identified from the ET tubes have been linked to endocarditis: *S. aureus*, *Granulicatella adiacens*, *H. parainfluenzae*, *Abiotrophia para-adiacens*, *N. perflava*, and *N. mucosa* (Christensen and Facklam, 2001; Forward, 2006; Kang et al., 2009; Saez Nieto et al., 1998). However, while each of these organisms are commonly linked to endocarditis, *S. aureus* was recently identified as one of the most common causes of endocarditis (Kang et al., 2009). *N. perflava* and *N. mucosa* have also been associated with meningitis (Saez Nieto et al., 1998). *F. nucleatum* and *P. nigrescens* are normal flora of the mouth, which can cause significant periodontal disease (Falagas and Siakavellas, 2000; Jacinto et al., 2008). Workers have suggested that *F. nucleatum* forms synergistic relationships with *Prevotella* spp. to increase their pathogenic potential within the oral cavity and other organs (Jacinto et al., 2008). The biofilm surrounding ET tubes A and G each contained sequences of *F. nucleatum* and *Prevotella* spp. In addition, ET tubes B and C also contained sequences at the genus level with >95% identity to *Fusobacterium* and *Prevotella*. Therefore, these synergistic microbes may become pathogenic for the patient populations due to their establishment in ET tube biofilms. Sequences similar to the potential pathogenic genus *Enterococcus* were only identified in the biofilm from ET tube G, which had been in place for 7 days. Urine from this patient cultured positive for *Enterococcus* spp. on the third day of intubation (Table 1), indicating systemic colonization with urinary tract infection. *Enterococcus* spp. are opportunistic pathogens found naturally within the human intestinal flora. *E. faecalis*, in particular, is associated with 80–90% of human enterococci infections (Mohamed and Huang, 2007) and sequences closely related to this species were found in ET tube G (Fig. 4B). It is likely that *E. faecalis* on this ET tube that was present for 7 days originated from the GI tract either through refluxed gastric fluids, colonized in the oral cavity followed by colonization of respiratory tract as reported previously (Bahrani-Mougeot et al., 2007), or externally from colonization of healthcare workers hands followed by ET tube manipulation.

Although methicillin-resistant *S. aureus* (MRSA) was identified by nasal PCR swabs or trachea aspirates in four of the patients by the Barnes-Jewish Hospital department of laboratories (ET tubes B, D, E, and H), only ET tubes D and H had high enough relative numbers of *S. aureus* to be cloned and sequenced with our gene survey method (Table 1). ET tube D was collected from a patient diagnosed with community-acquired pneumonia, while ET tube H came from a patient positively colonized with MRSA upon admission and with the highest number of ventilator days. ET tube biofilms B and E came from patients positive for MRSA by nasal PCR analysis upon admission, but correlating sequences were not found to be predominant within the sequenced biofilm extracts. This may be attributed to *S. aureus* being carried asymptotically within the anterior nares and on the skin (Wolf and Daley, 2007). MRSA detection on admission is used as an infection control measure to prevent the spread of hospital-acquired MRSA (Wenzel et al., 2008). Therefore, *S. aureus* within ET biofilms may not be determined solely by intubation duration, but a combination of intubation duration, days of hospitalization, and prior MRSA colonization.

Prolonged ET intubation periods with antibiotic treatment may support pathogenic proliferation

Nearly 95% (117/124) of the sequences collected from ET tube H were identified to be >97% identical to *P. aeruginosa*. This ET tube had been in place for 23 days, the longest of all the tubes collected. Trachea aspirate specimens from this patient were positive for *P. aeruginosa* through laboratory cultures 18 days after intubation (Table 1). *P. aeruginosa* is a ubiquitous Gram-negative

gammaproteobacterium found in water, soil, plants, and animal tissue (Wagner and Iglewski, 2008). Its genome is nearly twice the size of *E. coli* and includes genes for a variety of metabolic pathways enabling *P. aeruginosa* to sustain within diverse environments (Bjarnsholt and Givskov, 2007). The genome of *P. aeruginosa* includes an array of antimicrobial defenses and virulence factor offensives, making it a recalcitrant opportunistic pathogen for susceptible patients. In the hospital setting, *Pseudomonas* sp. infections are typically associated with cystic fibrosis patients, chronic wounds, and foreign objects inserted into the body on which biofilms may persist, including contact lenses, catheters, and ET tubes (Bjarnsholt and Givskov, 2007). The proliferation behavior of *P. aeruginosa* became apparent in ET tube H after a 23-day intubation period in conjunction with broad-spectrum antibiotic treatment, resulting in >95% of all sequences to be closely related to *P. aeruginosa*. Flanagan et al. (2007) showed that antibiotic usage is highly correlated to a decrease in microbial diversity of daily endotracheal aspirates from *P. aeruginosa*-colonized patients. Our work here supports such a loss of bacterial diversity in intubated patients colonized with *P. aeruginosa* and undergoing antibiotic treatment, with ET tube H having the lowest OTU number/non-chimeric sequences ratio compared to the other ET tubes (Table 1).

Understanding ET tube biofilms will help guide interventions to prevent VAP

ET tube biofilms are suspected in playing a part in the development of at least some VAP. Potential mechanisms of infection with bacteria from ET tubes include: relatively large biofilm pieces sloughing off and “falling” into the lower respiratory tract (Edwards, 2000); individual cells becoming aerosolized and aspirated into the lungs; individual cells being in liquid contact using motile capabilities to move deep into the lungs (Luna et al., 2009). The presence of the ET tube prevents the body's natural clearance mechanisms for preventing respiratory infections (Vincent, 2004). Although many of the organisms found in the biofilms in this study are not recognized as common pathogens causing VAP, in many cases patients with suspected VAP never have a pathogen identified. Further studies linking 16S rRNA sequences found on ET tubes with those found in tracheal aspirates or bronchoalveolar lavage specimens may clarify whether organisms from the biofilm cause some of these VAPs. In addition, the biofilm from the ET tube that had been in place for 23 days demonstrates that known pathogens can dominate the ET tube biofilm placing the patient at higher potential risk for VAP. ET tubes coated with antimicrobial agents have been developed (Kollef et al., 2008) to help mitigate biofilm formation. Similar tubes have been shown to have less biofilm when measured by culture in animal models (Berra et al., 2004). 16S rRNA surveys may provide a more sensitive way to evaluate the mechanism of this technology at reducing VAP.

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