Characterization of Bacterial Biofilms on Tracheostomy Tubes

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Objectives/Hypothesis: To characterize the structure and microbial content of biofilms found on tracheostomy tubes. To determine the correlation between the patients’ clinical condition and biofilm content.

Study Design: Prospective observational series.

Methods: Tracheostomy tubes were collected from patients in both the inpatient and outpatient setting at an urban academic medical center. Sections of the tracheostomy tubes were evaluated by confocal microscopy and bacteria from them plated and identified. The number of colony forming units (CFUs) and species present were determined and a univariate analysis performed to correlate them with various clinical factors.

Results: Bacteria were cultured from 19 of the 21 tracheostomy tubes collected. There were between $1 \times 10^6$ and $1 \times 10^{10}$ CFUs present in each of the 2 mm sections. Twelve different bacterial species and one fungus were isolated from culture and speciation. The number of bacteria isolated and the CFUs calculated varied in tubes obtained from the same patient at different times.

Conclusions: Biofilms were present on tracheostomy tubes in greater than 90% of tracheostomy tubes collected as early as 7 days after insertion in both the inpatients and outpatients. Although a variety of bacteria were identified in the biofilm, they often appeared as discrete microcolonies that appeared to be monospecies biofilm on confocal microscopy. There was a statistically significant inverse correlation between the number of colony forming units found and frequency of inner cannula change.

Key Words: Biofilm, tracheostomy tube, tracheostomy tube, colony forming units.

INTRODUCTION

Biofilms are complex three-dimensional structures composed of bacteria living in an extracellular matrix rich in polysaccharides, nucleic acids and proteins. Biofilm formation occurs in a well-defined series of steps. The bacteria first adhere to a surface. This surface can be either artificial, such as an implant, or natural, such as a tooth surface. Following attachment, bacteria aggregate and replicate to form microcolonies that produce the extracellular matrix resulting in a mature biofilm.

Within mature biofilms bacteria may enter a slow growth or quiescent state, allowing for prolonged survival. Some bacteria within biofilms will go through cycles of active shedding, whereas others are shed by shear forces. These intermittently shed bacteria could potentially lead to active infection and may be the cause of some recurring infections despite treatment with multiple courses of antibiotics. Biofilms have been implicated in recurrent septicemia in patients with indwelling devices, recurrent acute otitis media, recurrent Pseudomonas pneumonias in cystic fibrosis patients, and in infectious endocarditis.

Resistance mechanisms afforded by the presence of biofilms include decreased rates of bacterial division, which make antibiotics less effective. A negatively charged biofilm surface can repel positively charged antibiotic molecules. Differences in the microenvironment within the biofilm allow for differential gene expression that permit bacteria to better survive in high-stress, low-nutrient environments. Changes in membrane structure, such as increased expression of efflux pumps, decrease the accumulation of antibiotics in bacterial cells. Finally, the large size of the biofilm matrix makes it difficult for phagocytes to ingest them. These and other mechanisms make bacteria in biofilms resistant to antibiotics even at concentrations several thousand times greater than the minimum inhibitory concentration.
The presence of biofilms on indwelling biomedical devices, such as pressure equalization tubes, orthopedic prostheses, urinary catheters, intravascular catheters, and dental implants, has been increasingly recognized over the past decade.\(^6\)\(^,\)\(^7\) Biofilm formation on tracheostomy tubes has been less well studied. Jarrett et al. observed biofilm formation on tracheostomy tubes in vitro after inoculating tracheostomy tubes made of four different materials with *Pseudomonas aeruginosa* and *Staphylococcus epidermidis*.\(^8\) Perkins et al. noted an increasing density of biofilm formation as one progressed toward the tip of tracheostomy tubes obtained from non-ventilated pediatric inpatients.\(^9\)

We undertook this study to characterize biofilm formation on tracheostomy tubes in adults by determining their structure using confocal microscopy, specifying the types and quantifying the number of bacteria present, and correlating this with the patients’ clinical condition at the time of tube change.

**MATERIALS AND METHODS**

After obtaining approval from our university’s institutional review board, both inpatients and outpatients at Temple University Hospital and the Department of Otolaryngology–Head and Neck Surgery requiring tracheostomy tube replacement were asked to enroll in this study. The patients’ medical and social history was reviewed and recorded (Table I). The type of tracheostomy tube, time since last change, and appearance of the stoma were also recorded at the time of tube change.

The exterior of the tracheostomy tubes was first wiped with ethanol to remove and kill any bacteria present on the outside surface. The tube was then sterilely sectioned into 2-mm slices in a tissue culture hood to prevent contamination; two adjacent sections were used for analysis. One section was fixed in 3.7% formaldehyde, stained with Syto7 (Molecular Probes Inc., Portland, OR), which fluorescently labels DNA, and then imaged using an inverted Leica TSS confocal microscope (Leica Microsystems, Wetzlar, Germany). The adjacent section of the tube was placed in 10 mL of phosphate buffered saline (PBS) and sonicated to release bacteria using a Fisher Scientific Sonic Dismembrator Model 500 (Thermo Fisher Scientific Inc., Waltham, MA) at 60% for three 10-second pulses. One hundred microliters of this fluid were then diluted 1:1000 in PBS, plated on tryptic soy agar (TSA) or TSA supplemented blood agar (BAP) and incubated overnight at 37°C. On the following morning, the plates were visually inspected for number and types of colonies present. Images of the plates were then captured using an Epson digital scanner (Epson America Inc., Long Beach, CA) for examination and quantification of colonies.

The number of colony forming units (CFUs) was then calculated for each 2 mm section, with \(10^6\) being the lower limit of detection. Representative colony types were chosen at random from at least three independent plates for inoculation in tryptic soy broth. After overnight incubation at 37°C, samples were removed, mixed with 30% glycerol and stored at \(-70°C\).

Precise determination of all bacterial species present based upon plating alone was difficult. Although some bacterial colonies such as *Proteus vulgaris* had a very distinct morphology, others, including coagulase-negative staphylococci and *Staphylococcus aureus*, could not be reliably distinguished by morphology alone on TSA or BAP without further diagnostic testing. Colonies were selected for speciation from 14 of the 19 specimens. Because the difficulty in speciating bacteria based on colony morphology alone was noted late in the study, bacterial stocks did not exist for five of the 19 positive specimens, and not all of the bacteria species from a single patient were available as stocks.

The 14 stored stocks were then further purified by serial plating, Gram stained, and speciated by standard clinical microbiology laboratory methods using pure cultures. Gram-positive organisms were evaluated by catalase testing (Fisher Chemicals, Fair Lawn, NJ) and slide coagulase testing (Pastorex Staph Plus, Bio-Rad, Marnes La Coquette, France). *Streptococcus* species were identified by the RapID STR System (Remel, Inc., Lenexa, KS). *Enterococcus* species were identified by PYR

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**TABLE I.**

| Patient Identifier | Ventilator Use, Yes/No | Hypertension | Obstructive Sleep Apnea | COPD | Asthma | Diabetes |
|--------------------|------------------------|--------------|------------------------|------|--------|---------|
| 1                  | Yes                    | Yes          | Yes                    | No   | No     | No      |
| 2                  | No                     | No           | No                     | No   | No     | No      |
| 3                  | No                     | No           | No                     | No   | No     | No      |
| 4                  | Yes                    | Yes          | Yes                    | No   | No     | No      |
| 5                  | Yes                    | Yes          | No                     | No   | No     | No      |
| 6                  | Yes                    | Yes          | No                     | No   | No     | No      |
| 7                  | Yes                    | Yes          | No                     | No   | No     | No      |
| 8                  | Yes                    | Yes          | Yes                    | No   | No     | No      |
| 9                  | No                     | No           | No                     | No   | No     | No      |
| 10                 | No                     | Yes          | Yes                    | No   | No     | No      |
| 11                 | Yes                    | Yes          | No                     | No   | No     | No      |
| 12                 | Yes                    | Yes          | No                     | No   | No     | No      |
| 13                 | No                     | No           | No                     | No   | No     | No      |
| 14                 | No                     | No           | No                     | No   | Yes    | No      |

COPD = chronic obstructive pulmonary disease.
*Inpatient during tracheostomy tube harvest.
testing (Remel, Inc.) and a Becton Dickinson Phoenix automated system PMIC/ID 102 (Becton Dickinson Co., Sparks, MD). Gram-negative organisms were identified with the Becton Dickinson Phoenix automated system NMIC/ID 127 (Becton Dickinson Co.).

A univariate regression analysis evaluating colony forming units (CFUs) in logarithmic units as compared to multiple data points from the patient’s past medical history and tracheostomy care-related factors was carried out. Analysis was evaluated using SAS version 9.1 statistics software (SAS Corporate Statistics, Cary, NC).

RESULTS

Twenty-one tracheostomy tubes were collected from 14 patients between May and October 2008. Two of the tracheostomy tubes were collected from hospital inpatients; the remaining 19 tracheostomy tubes were collected from 12 different patients in the outpatient setting. The age of the patients enrolled in this study ranged from 21 years to 83 years with a mean of 55 years and a median of 60 years. There were seven males and seven females.

Four patients were mechanically ventilated at some point during the day. Three patients were active tobacco smokers and four of the patients were active alcohol drinkers. Seven patients had hypertension, five had diabetes, two patients had obstructive sleep apnea, two had previous cerebrovascular accidents, two had gastroesophageal reflux, one had asthma, one had a seizure disorder, one was schizophrenic, and one had a past myocardial infarction.

Indications for tracheostomy in the patient population studied were ventilator-dependent respiratory failure in four patients, tracheal stenosis in two patients, laryngeal stenosis in two patients, laryngeal cancer in one patient, obstructive sleep apnea in one patient, chondromalacia in one patient, and chronic aspiration following cerebrovascular accident in one patient. Four patients were mechanically ventilated at some point during the day.

Of the 21 tracheostomy tubes collected, 17 were uncuffed Shiley tubes, two were cuffed Shiley tubes, one was an uncuffed Bivona Hyperflex tube, and one was an uncuffed Portex tracheostomy tube. Stomal edema, induration, or granulation tissue was noted in six patients at eight of the 21 tracheostomy tube changes performed. Patients enrolled in this study reported changing their inner cannula, if present, from one to seven times per day. Recent sputum culture results were present in three of 21 patients at the time of tracheostomy tube change. In two cultures, methicillin resistant Staphylococcus aureus was present and Candida albicans was present in another.

Biofilm formation on the tracheostomy tube sections was observed using confocal microscopy (Figs. 1 and 2). Biofilms appeared to form both on the tube surface and in the mucus layer coating the inside of the tube. In the latter, the biofilms often appeared as discrete

### TABLE II.
Tracheostomy Tube Data.

| Patient Identifier | Collection Date | Duration Tracheostomy Tube in Place, d | Type of Tracheostomy | ID of Tube, mm | Cuffed or Uncuffed | Stomal Edema | Stomal Induration | Stomal Granulation Tissue | Times Inner Cannula Changed Daily |
|--------------------|-----------------|----------------------------------------|----------------------|---------------|-------------------|--------------|------------------|---------------------------|----------------------------------|
| 1                  | 9/8/08          | 162                                    | Shiley DCFS          | 8             | Uncuffed          | Yes          | Yes              | Yes                       | 7                                |
| 2                  | 8/19/08         | 70                                     | Portex              | 7             | Uncuffed          | No           | No               | No                        | 1                                |
| 3                  | 8/18/08         | 61                                     | Shiley DCFS          | 4             | Uncuffed          | No           | No               | No                        | 3                                |
| 4                  | 9/15/08         | 87                                     | Shiley DCFS          | 6             | Uncuffed          | No           | No               | No                        | 1                                |
| 5                  | 6/17/08         | 64                                     | Shiley XLT           | 6             | Uncuffed          | No           | No               | Yes                       | 3                                |
| 6                  | 8/19/08         | 62                                     | Shiley XLT           | 6             | Uncuffed          | No           | No               | No                        | 3                                |
| 7                  | 5/12/08         | 120                                    | Shiley XLT           | 6             | Uncuffed          | Yes          | Yes              | Yes                       | 1                                |
| 8                  | 6/2/08          | 50                                     | Bivona Hyperflex     | 6             | Uncuffed          | No           | No               | No                        | Not applicable                   |
| 9                  | 6/2/08          | 68                                     | Shiley DCFS          | 6             | Uncuffed          | No           | No               | No                        | 1                                |
| 10                 | 5/19/08         | 41                                     | Custom Shiley        | 7             | Uncuffed          | Yes          | Yes              | Yes                       | Not applicable                   |
| 11                 | 5/12/08         | 55                                     | Custom Shiley        | 7             | Uncuffed          | Yes          | Yes              | Yes                       | Not applicable                   |
| 12                 | 8/19/08         | 49                                     | Shiley DCT           | 6             | Cuffed            | No           | Yes              | No                        | 3                                |
| 13                 | 5/8/08          | 22                                     | Shiley XLT           | 6             | Uncuffed          | No           | No               | No                        | 2                                |
| 14                 | 7/7/08          | 67                                     | Shiley DCFS          | 4             | Uncuffed          | No           | No               | No                        | 1                                |
| 15                 | 10/13/08        | 21                                     | Shiley DCFS          | 4             | Uncuffed          | No           | No               | No                        | 2                                |

ID = inner diameter.
microcolonies that appeared to be monospecies biofilms even though there were multiple types of bacteria present on the tracheostomy tube. Biofilm formation was detected as early as 1 week after tube insertion, which was the earliest time point evaluated.

Bacteria were cultured from 19 of the 21 tracheostomy tubes collected. There were between $1 \times 10^6$ and $1 \times 10^{10}$ CFUs present in each of the 2-mm sections. The numbers were evenly distributed across the range. Patients 6 and 13 both had no detectable bacteria on their tracheotomy tubes with $1 \times 10^6$ CFUs being the lower limit of detection. The number of bacteria isolated and the CFUs calculated varied in tubes obtained from the same patient at different times.

Twelve different bacterial species and one fungus were isolated from culture and speciation. Bacterial species isolated included *Staphylococcus aureus*, coagulase negative *Staphylococcus aureus*, *Escherichia coli*, *Enterococcus fecalis*, *Streptococcus mitis*, *Serretia marcescens*, *Stenotrophomonas maltophilia*, diphtheroid bacteria, *Proteus vulgaris*, *Pseudomonas aeruginosa*, beta-hemolytic group B streptococci, and *Citrobacter koseri*, whereas *Candida albicans* was the sole fungus isolated (Table III).

Using a univariate regression analysis, an inverse correlation was noted between the number of CFUs present in each 2 mm tracheostomy tube section and the number of times the tracheostomy inner cannula was changed daily ($P = .0137$). None of the other aspects of the past medical history nor tracheostomy care-related factors had a statistically significant correlation (Table IV).

**DISCUSSION**

Biofilms are complex, organized, three-dimensional structures that may form on indwelling medical devices as quickly as 2 hours after insertion, although some bacteria take weeks to form biofilms on these devices depending upon environmental factors. In our study, biofilms were present at the earliest time point evaluated, 7 days after placement of a tracheostomy tube. These findings were consistent with those of Jarrett et al., where biofilm was noted on tracheostomy tubes after 6 days of intubation in a laboratory setting.

The presence of biofilms on 19 of 21 tracheostomy tubes is in keeping with Perkins, et al.’s findings on pediatric tracheostomy tubes in which 10 of 11 pediatric...
tubes were noted to have biofilms present by confocal microscopy with concurrent bacterial staining.9 Unique to our study, however, was that the biofilms in the mucus layer appeared to be monospecies biofilms. When multiple bacterial species are present in a biofilm, the bacteria will grow either intermixed or in close proximity to each other. In the mucus layer, the biofilms seemed to be separate monospecies biofilms. This may be due to factors unique to biofilm formation in mucus or properties of the bacteria forming the biofilms.

The absence of detectable bacteria in two of our patients may well be related to excellent local care. Both patients who did not have biofilms identified in their tracheostomy tubes at the time of collection reported being fastidious with their tracheostomy care, including removing and cleaning the entire tube multiple times per week.

The organisms identified in our study include many of the common flora of the upper aerodigestive tract and have been isolated from biofilms on endotracheal tubes and laryngeal stents.11 Though the speciation for each patient may not be complete, some trends were

### TABLE III.
Microbial Content of Tracheostomy Tubes.

| Patient | Date of Sample | CFU/2 mm of Tube on TSA* | Species Identified† |
|---------|----------------|--------------------------|---------------------|
| 1       | 9/8/08         | $1.0 \times 10^6$        | *Staphylococcus aureus* |
| 2       | 8/19/08        | $6.6 \times 10^7$        | *Serratia marcescens, Stenotrophomonas maltophilia, Diphtheroid bacteria* |
| 3       | 8/18/08        | $4.5 \times 10^7$        | *Proteus vulgaris, Pseudomonas aeruginosa, Candida albicans* |
| 4       | 9/15/08        | $2.5 \times 10^9$        | *Escherichia coli, Enterococcus faecalis* |
| 5       | 6/17/08        | $4.7 \times 10^7$        | *Escherichia coli, Staphylococcus aureus* |
| 5       | 8/19/08        | $3.3 \times 10^8$        | Coagulase-negative staphylococci, *Escherichia coli*, beta-hemolytic group B streptococci, *Staphylococcus aureus* |
| 5       | 10/20/08       | $4.2 \times 10^8$        | *Citrobacter koseri* |

CFU = colony forming units; TSA = tryptic soy agar; ND = no colonies were detected (lower limit of detection $1 \times 10^6$ bacteria per 2-mm section).

*CFU per 2 mm of tracheostomy tube as determined by plating on TSA agar.

†Single colonies from either the TSA plate or BAP were chosen and submitted to the diagnostic laboratory of Temple University Hospital for identification. It is important to note that for any individual patient, each species represents a colony chosen for identification and may not represent all of the bacterial species present in the patient.

### TABLE IV.
Univariate Analysis of CFU Versus Patient History/Tracheostomy Care Factors.

| Effect                     | P Value |
|----------------------------|---------|
| Tube duration               | .3966   |
| Tube type                   | .2909   |
| Tube size                   | .3503   |
| Tube cuff                   | .9326   |
| Stomal edema                | .6621   |
| Stomal induration           | .4134   |
| Stomal granulation          | .6524   |
| Inner cannula changes       | .0137   |
| Vent use                    | .6393   |
| Hypertension                | .5529   |
| OSA                         | .897    |
| COPD                        | .9326   |
| Asthma                      | .7182   |
| Diabetes                    | .5736   |

CFU = colony forming units; OSA = obstructive sleep apnea; COPD = chronic obstructive pulmonary disease.

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identified. The species of bacteria isolated were variable from patient to patient and over time in the same patient. Coagulase negative staphylococci were isolated from a number of patients. The predominant coagulase negative staphylococci is *Staphylococcus epidermidis*. *Staphylococcus epidermidis* is often a member of the normal flora and an excellent biofilm former, which may account for its presence in six of the 12 tracheostomy tubes on which speciation was completed. *Staphylococcus aureus* was present in four patients in whom bacterial species were identified, and three of these patients had evidence of stomal granulation. Grillo et al. observed stomal granulation tissue in the presence of nonabsorbable suture and correlated this with the presence of *Pseudomonas aeruginosa* and *Staphylococcus aureus* on culture. The presence of *Escherichia coli*, which is a normal fecal flora, in two patients confirms that hygiene may contribute to colonization and subsequent biofilm formation.

One patient exhibited beta-hemolytic group B streptococci, suggesting that it can form biofilms in mucus or on tracheostomy tubes. Although *Streptococcus agalactiae* biofilm formation is believed to occur during bovine mastitis, there has been only one report of group B streptococci growing in a biofilm. This was present on three different tubes collected from this patient and appeared to have a two to three logarithm increase in the number of colony forming units at one point, suggesting a possible infection.

Several patients in our study had *Pseudomonas aeruginosa*. Although *Pseudomonas aeruginosa* is often associated with ventilator assisted pneumonia (VAP), no patient in our study had an acute pulmonary infection. The question remains whether bacterial biofilms within endotracheal tubes and tracheostomy tubes are causative agents for VAP, or if the bacteria present in these biofilms are merely colonizing tracheostomy tubes after an active infection has cleared.

Prior to this study, quantification of bacterial CFUs within tracheostomy tube biofilms had not been reported. The number of CFUs ranged from $1 \times 10^6$ to $1 \times 10^{10}$ in those tracheostomy tubes that had biofilms present. Of note, in one patient, an increase in the CFU count was associated with a hospital admission and correlated with a bacterial tracheitis, later confirmed to be due to methicillin resistant *Staphylococcus aureus*. The univariate regression analysis performed was also able to show a negative correlation between the number of CFUs present and the number of times an inner cannula was changed daily, again confirming the importance of local care.

**CONCLUSION**

Bacterial biofilms are present in greater than 90% of tracheostomy tubes collected in both the inpatient and outpatient setting. Although a variety of bacteria were identified in the biofilm, they often appeared as discrete microcolonies that appeared to be monospecies biofilm on confocal microscopy. The number of colony forming units was between $1 \times 10^6$ and $1 \times 10^7$ per 2-mm section, which inversely correlated with the frequency of the inner cannula change ($P < .05$).

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**BIBLIOGRAPHY**

1. Sternberg C, Christensen BB, Johansen T, et al. Distribution of bacterial growth activity in flow-chamber biofilms. *Appl Environ Microbiol* 1999;65:4108–4117.
2. Saye DE. Recurring and antimicrobial-resistant infections: considering the potential role of biofilms in clinical practice. *Ostomy Wound Manage* 2007;53:46–62.
3. Hall-Stoodley L, Hu FZ, Gieseke A, Nistico L, et al. Direct detection of bacterial biofilms on the middle-ear mucosa of children with chronic otitis media. *JAMA* 2006;296:202–11.
4. O’Gara JP, Humphreys H. *Staphylococcus epidermidis* biofilms: importance and implications. *J Med Microbiol* 2001;50:582–587.
5. Anderl JN, Franklin MJ, Stewart PS. Role of antibiotic penetration limitation in *Klebsiella pneumoniae* biofilm resistance to ampicillin and ciprofloxacin. *Antimicrob Agents Chemother* 2000;44:1818–1824.
6. Cristina AG, Costerton JW. Bacterial adherence to biomaterials and tissue. The significance of its role in clinical sepsis. *J Bon Joint Surg* 1985;67:264–273.
7. Donlan RM, Costerton JW. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin Microbiol Rev* 2002;15:167–193.
8. Jarrett W, Ribes J, Manaligod J. Biofilm formation on tracheostomy tubes. *Ear Nose Throat J* 2002;81:650–661.
9. Perkins J, Mouzakes J, Pereira R, Manning S. Bacterial biofilm presence in pediatric tracheotomy tubes. *Arch Otolaryngol Head Neck Surg* 2004;130:339–343.
10. Jones SM, Morgan M, Humphrey TJ, Lappin-Scott H. Effect of vancomycin and rifampin on methicillin-resistant *Staphylococcus aureus* biofilms. *Lancet* 2001;357:40–41.
11. Payman S, Wiatrak BJ. Microbiology of stents in laryngotracheal reconstruction. *Laryngoscope* 2004;114:364–367.
12. Grillo HC, Zannini P, Michelassi F. Complications of tracheal reconstruction. *J Thorac Cardiovasc Surg* 1986;91:322–328.
13. Olson ME, Ceri H, Morck DW, Buret AG, Read RR. Biofilm bacteria: formation and comparative susceptibility to antibiotics. *Can J Vet Res* 2002;66:86–92.