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Sphingosine Prevents Bacterial Adherence to Endotracheal Tubes: A Novel Mechanism to Prevent Ventilator-Associated Pneumonia

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14. ABSTRACT
Ventilator-associated pneumonia (VAP) is one of the most common nosocomial infections causing significant morbidity and mortality in critically ill patients. VAP prevention is an area that has received much attention. Bacteria responsible for VAP are diverse; however, 60% of causative agents are Gram-negative bacteria, most commonly *Pseudomonas aeruginosa* (PA) and *Acinetobacter baumannii* (AB). The cause of VAP is multifactorial, but is undeniably related to the presence of the endotracheal tube (ETT). Tracheal intubation inhibits the cough reflex, affects mucociliary clearance, provides direct access for bacteria from the upper to the lower respiratory system, and allows for the formation of biofilm, which can subsequently be a source of persistent infection. Thus, prevention of bacterial adherence on the surface of ETTs could have potential to significantly reduce rates of VAP. In this study, we used an in vitro bacterial adherence model to study the effectiveness and durability of sphingosine and phytosphingosine coating to reduce bacterial adherence of AB, PA, and *Staphylococcus aureus* to the surface of ETTs compared to standard plasticized polyvinyl chloride and silver-coated ETTs. Our data show that sphingosine-coated and phytosphingosine-coated ETTs are highly efficacious at preventing bacterial adherence against three of the most common pathogens, AB, PA, and *Staphylococcus aureus*, associated with VAP when compared with standard plasticized polyvinyl chloride ETTs. Additionally, our data indicate our sphingolipid-coated tubes are equally efficacious as silver-coated ETTs. Future in vitro and animal studies are necessary to establish the safety of sphingolipid coatings, and future randomized clinical trials will be necessary to determine sphingosine or phytosphingosine's ability to provide a cost-effective preventative strategy to reduce rates of VAP.

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1.0 SUMMARY

Ventilator-associated pneumonia (VAP) is one of the most common nosocomial infections causing significant morbidity and mortality in critically ill patients. VAP prevention is an area that has received much attention. Bacteria responsible for VAP are diverse; however, 60% of causative agents are Gram-negative bacteria, most commonly *Pseudomonas aeruginosa* (PA) and *Acinetobacter baumannii* (AB). The cause of VAP is multifactorial, but is undeniably related to the presence of the endotracheal tube (ETT). Tracheal intubation inhibits the cough reflex, affects mucociliary clearance, provides direct access for bacteria from the upper to the lower respiratory system, and allows for the formation of biofilm, which can subsequently be a source of persistent infection. Thus, prevention of bacterial adherence on the surface of ETTs could have potential to significantly reduce rates of VAP. In this study, we used an in vitro bacterial adherence model to study the effectiveness and durability of sphingosine and phytosphingosine coating to reduce bacterial adherence of AB, PA, and *Staphylococcus aureus* to the surface of ETTs compared to standard plasticized polyvinyl chloride and silver-coated ETTs. Our data show that sphingosine-coated and phytosphingosine-coated ETTs are highly efficacious at preventing bacterial adherence against three of the most common pathogens, AB, PA, and *Staphylococcus aureus*, associated with VAP when compared with standard plasticized polyvinyl chloride ETTs. Additionally, our data indicate our sphingolipid-coated tubes are equally efficacious as silver-coated ETTs. Future in vitro and animal studies are necessary to establish the safety of sphingolipid coatings, and future randomized clinical trials will be necessary to determine sphingosine or phytosphingosine's ability to provide a cost-effective preventative strategy to reduce rates of VAP.

2.0 INTRODUCTION

Ventilator-associated pneumonia (VAP) is one of the most common nosocomial infections causing significant morbidity and mortality in critically ill patients. It affects up to 25% of mechanically ventilated patients and has a 13% mortality [1]. VAP adds 5-7 days to intensive care unit (ICU) length of stay and increases the length of hospitalization by 10-12 days [2].

VAP prevention is an area that has received much attention. VAP bundles, evidence-based practices that have been identified and implemented to reduce rates of VAP, include semi-recumbent positioning, daily wake-wean trials, small bowel tube feeding, prophylactic probiotics, and early tracheostomy [1,3,4]. Evidence has shown silver-coated endotracheal tubes (ETTs) to be effective at reducing rates of VAP by 36% [1,5]. However, silver-coated tubes are not seen in routine hospital use and are not included in the National Guideline Clearinghouse Prevention of Ventilator-Associated Pneumonia provided by the Agency for Healthcare Research and Quality, part of the U.S. Department of Health and Human Services. A potential reason for this lack of implementation is the large upfront cost of implementing silver-coated ETTs, as they can be up to 100 times more expensive than standard plasticized polyvinyl chloride (PVC) tubes.

Bacteria responsible for VAP are diverse; however, 60% of causative agents are Gram-negative bacteria, most commonly *Pseudomonas aeruginosa* (PA) and *Acinetobacter baumannii* (AB). The most common Gram-positive bacteria involved in VAP is *Staphylococcus aureus* (SA) [6]. The cause of VAP is multifactorial, but is undeniably related to the presence of the ETT. Tracheal intubation inhibits the cough reflex, affects mucociliary clearance, provides direct

access for bacteria from the upper to the lower respiratory system, and allows for the formation of biofilm, which can subsequently be a source of persistent infection [7].

Biofilms are increasingly becoming an area of focus secondary to their roles in chronic or persistent infections often resistant to standard antibiotic therapy [8]. Biofilm can be defined as "a coherent cluster of bacterial cells imbedded in a biopolymer matrix, which, compared with planktonic cells, shows increased tolerance to antimicrobials and resists the antimicrobial properties of the host defense" [9]. Bacterial adhesion is the first step in biofilm formation [10]; thus, prevention of bacterial adherence on the surface of ETTs could have potential to significantly reduce rates of VAP.

Sphingolipids are a group of lipids that modulate multiple cellular functions, including cell proliferation, cycle regulation, inflammatory pathways, and apoptosis. Sphingosine is a sphingoid long chain base that is generated from ceramide using the enzyme (acid) ceramidase. Sphingosine has been previously shown to have antimicrobial properties against both Gram-positive and Gram-negative bacteria [11]. It has been described as an integral part of the innate immunity of the skin [12], oral mucosa [13], and respiratory mucosa [14].

In this study, we used an in vitro bacterial adherence model to study the effectiveness and durability of sphingosine and phytosphingosine coating to reduce bacterial adherence of AB, PA, and SA to the surface of ETTs compared to standard plasticized PVC and silver-coated ETTs.

3.0 MATERIALS AND METHODS

3.1 Materials

D-erythrosphingosine (d18:1) and D-ribo-phytosphingosine were purchased from Avanti Polar Lipids (Alabaster, AL). Hexane (anhydrous, 95%) and acetone (>99.9%) were purchased from Sigma-Aldrich. Absolute, 200 proof, molecular biology grade ethanol was purchased from Fisher Scientific (Pittsburgh, PA). Plasticized PVC ETTs (8.0 mm) were purchased from Cardinal Health (Dublin, OH). Silver-coated ETTs (8.0 mm) were purchased from Bard Medical (Covington, GA). Plastic coverslips (24 x 60 mm) were purchased from Electron Microscopy Sciences (Hatfield, PA). Three different bacterial strains were used: methicillin-resistant *Staphylococcus aureus* (MRSA) (USA 300), *Acinetobacter baumannii*, and *Pseudomonas aeruginosa* (ATCC [American Type Culture Collection], Manassas, VA).

3.2 Evaporative-Induced Deposition of Sphingolipids

Sphingolipid solutions were prepared by dissolving either sphingosine or phytosphingosine into organic solvents (i.e., hexane, acetone, or ethanol). The concentration of sphingosine in hexane was 30 mM. Sphingosine was added to hexane, which was preheated to 60°C in a water bath. After addition of sphingosine, the solution was agitated and sonicated until the sphingosine aggregates were no longer visible and the solution was clear. Phytosphingosine (30 mM) in acetone and sphingosine (80 mM) and phytosphingosine (80 mM) in ethanol were prepared similarly with different heating temperatures. The preheated temperatures were 50°C and 70°C for acetone and ethanol, respectively.

Both ETT segments and plastic coverslips were used as coating substrates. ETT segments were prepared by cutting 1-cm-long sections of ETTs. Full-length plastic coverslips were utilized. Sphingolipid thin films were deposited onto the surface of ETTs and plastic coverslips

by dip coating the object into a heated sphingolipid solution. The tube segments were manipulated using a 1-mL insulin needle stuck through the plastic and the coverslips were manipulated using straight Kelly forceps. The tube segments/coverslips were immersed into the solution for 1 second and then slowly withdrawn at a rate of 1 cm/s. Evaporative-induced deposition of the thin film occurred when the object was then subjected to room temperature atmosphere. Of note, the plastic coverslips were less resistant to acetone and began to dissolve when dip coated; thus, we experimented with 100% ethanol as the solvent. Ethanol proved to work equally well, and since it is less likely to affect the structural integrity of the PVC tubes, we changed our coating process to use ethanol as our preferred solvent. Repeated dips of up to 10 times were utilized initially, but after coating optimization with ethanol as solvent, only 1 dip was necessary.

3.3 In Vitro Biofilm Colonization: Complete Immersion Assay

ETT segments were tested for inhibition of bacterial adherence using a modified version of the biofilm colonization model developed by Kuhn et al. [15] and utilized by Raad et al. [16]. Bacteria were grown overnight on trypticase soy agar (AB, PA) or trypticase soy agar with 5% sheep's blood (SA, MRSA) plates at 37°C. Bacterial suspensions were prepared by placing bacteria into 10 mL trypticase soy broth (TSB) (BD Biosciences) with sterile cotton tip applicators, diluting 1:10 in TSB, measuring absorbance at 550 nM, and diluting with TSB using standard curves prepared for each bacterial strain to achieve 500 colony forming units (cfu)/mL concentration. Sphingolipid-coated, uncoated, or vehicle-coated ETT segments were immersed in 2 mL bacterial suspension placed in 24-well plates and incubated for 12 hours at 37°C. ETT segments were rinsed in 100 mL HEPES/saline (H/S) (132 mM NaCl [sodium chloride], 20 mM HEPES [pH 7.4], 5 mM KCl [potassium chloride], 1 mM $CaCl_2$ [calcium chloride], 0.7 mM $MgCl_2$ [magnesium chloride], 0.8 mM $MgSO_4$ [magnesium sulfate]) at 37°C then agitated at 125 rpm for 30 minutes. Segments were then placed in 10 mL sterile H/S in test tubes and sonicated at 37°C in a bath sonicator for 10 minutes to remove adherent bacteria. Test tubes were vortexed for 5 seconds and the H/S serially diluted, plated on Lennox broth plates, and incubated overnight. Bacterial colony forming units were counted and the total amount of bacteria adherent to the 1-cm ETT segments was calculated.

3.4 In Vitro Biofilm Colonization: Variation on ISO 22196 and Durability Testing

Plastic coverslips were tested for inhibition of bacterial adherence using a modified version of the international standard for measurement of antibacterial activity on plastics and other non-porous surfaces, ISO 22196. Bacteria were prepared as described above to a concentration of 1 x 106 cfu/mL. Ten µL (10,000 cfu) of bacterial suspension was then placed on the sphingolipid-coated and ethanol-coated portion of the coverslips and covered with a 2-cm x 3-cm low density polyethylene (LDPE) plastic film and incubated for 24 hours at 37°C. The plastic film was removed and the plastic coverslips rinsed to remove planktonic bacteria. The coverslips were then placed into a drying rack and exposed to humidified air by incubating at 37°C for 12 hours. Coverslips were placed into 10 mL sterile H/S in test tubes and sonicated at 37°C in a bath sonicator for 10 minutes to remove adherent bacteria. Test tubes were vortexed for 5 seconds and the H/S serially diluted, plated on Lennox broth plates, and incubated

overnight. Bacterial colony forming units were counted and the total amount of bacteria adherent to the coverslips was calculated.

To study the durability of the coating against bacterial adherence, we inoculated the coated portion of the coverslip with additional bacteria after 24 and 48 hours. Bacteria were prepared the same as initial inoculation (1 x 106 cfu/mL). The LDPE plastic film was lifted, 10 µL (10,000 cfu) was pipetted onto the coated surface, and the LDPE film was replaced. The coverslips were incubated again for 24 hours, and the inoculation was again repeated at 48 hours. At 72 hours, the coverslips were H/S rinsed to remove planktonic bacteria, placed in drying racks and incubated for 12 hours, sonicated in sterile H/S for 10 minutes, diluted, and plated and quantification of adherent bacteria was performed.

To visualize the adherent bacteria after 24 hours, several coverslips were stained as follows prior to the removal of adherent bacteria by sonication. Coverslips were removed from the incubator after being rinsed and dried in the humidifier for 12 hours. The adherent bacteria were heat fixed to the coverslips by quickly passing over a Bunsen burner. The coverslips were then immersed in crystal violet for 1 minute, serially rinsed in H_2O, and mounted with VectaMount permanent mounting media. Slides were imaged using standard light microscopy.

3.5 In Vitro Biofilm Colonization: Air Exposure after Complete Immersion

Quantifying adherent bacteria after complete immersion in an aqueous bacterial suspension is not the best in vitro characterization of adherent bacteria on an ETT in an intubated patient. ETTs in vivo may be adjacent to respiratory epithelial cells and a thick mucous layer, but are also primary exposed to humidified air. To more closely replicate the in vivo conditions, we developed a variation of our methods described above in 3.3. ETT segments were coated as described in 3.2. Bacterial suspensions were prepared and ETT segments were placed into 24-well plates as described in 3.3. After 24 hours of incubation, however, the ETT segments were rinsed in 50 mL sterile H/S, suspended in air by sticking them with a 1-mL insulin needle, and incubated in humidified air at 37°C for 24 hours. Adherent bacteria were released from the surface and quantified as described in 3.3.

3.6 Characterization of Sphingolipid Coating of ETTs

ETT segments were dip coated with sphingolipid vs. vehicle as described in 3.2 to form a thin film coating. Samples were prepared as follows: coated segments were stained with 0.1% osmium tetroxide (Sigma) in H_2O for 30 minutes then rinsed in H_2O for 5 minutes. Segments were dried and transported to the scanning electron microscopy (SEM) lab. Segments were cut to fit on standard aluminum specimen mounts and placed on the mounts using conductive tape. Mounted segments were then sputter coated with gold/platinum for 15 seconds and imaged using SEM (FEI/Phillips XL30 SEM, Hillsboro, OR). To study the durability of the coating in aqueous solutions, samples were immersed in H_2O, H/S, or PBS for either 12 hours or 7 days at 37°C. Samples were then stained and imaged as described above.

3.7 Quantification of Sphingolipid on ETTs: Mass Spectrometry

Coated plastic surface was extracted by a one-step lipid extraction. Briefly, a plastic piece was transferred into a siliconized glass tube and adsorbed sphingosine was extracted by addition

of 10 mL methanol and sonification on ice for 1 hour. After centrifugation, the lipid extract was diluted with methanol and 50 pmol of C17-sphingosine was added as internal standard. Sphingosine species were separated by reverse-phase high performance liquid chromatography (HPLC) (Agilent 1260 series, Agilent Technologies, Waldbronn, Germany) using a Waters X-Bridge C18 separation column (4.6 mm × 150 mm, 3.5-µm particle size, 138-Å pore size) with a Waters X-Bridge C18 guard column (4.6 × 20 mm; Waters, Eschborn, Germany). Solvent A was 50:50 methanol-acetonitrile with 0.1% formic acid and solvent B was water with 0.1% formic acid. The gradient was increased from 72% A to 100% A between 0 minutes and 6 minutes, held at 100% A between 6 and 12 minutes, returned to 72% A between 12 and 13 minutes, and held at 72% A for 4 minutes to allow column reequilibration. The flow rate was 0.6 mL/min between 0 minutes and 5 minutes, increased from 0.6 mL/min to 1.0 mL/min between 5 minutes and 6 minutes, held at 1.0 mL/min between 6 minutes and 12 minutes, and returned to 0.6 mL/min between 12 and 13 minutes. The HPLC column effluent was introduced onto an Agilent 6490 triple quadrupole mass spectrometer (Agilent Technologies) operating in the positive electrospray ionization mode. The following ion source parameters were used: drying gas temperature 290°C, drying gas flow 11 L/min of nitrogen, sheath gas temperature 380°C, sheath gas flow 12 L/min nitrogen, nebulizer pressure 35 psi, capillary voltage 4500 V. Ion funnel parameters were as follows: high pressure radio frequency voltage 110 V and low pressure radio frequency voltage 60 V. Multiple reaction monitoring transition 300.3 m/z > 282.3 m/z at 8 eV collision energy was used for quantification of sphingosine, whereas multiple reaction monitoring transition 286.3 m/z > 268.3 m/z at 8 eV collision energy was used for quantification of C17-sphingosine. Quantification was performed with Mass Hunter Software (Agilent Technologies).

3.8 Quantification of Sphingolipid on ETTs: Sphingosine Kinase Assay

Plastic pieces were extracted in chloroform ($CHCl_3$)/methanol (CH_3OH)/1N hydrochloric acid (HCl) (100:100:1, v/v/v); the lower phase was dried and resuspended in a detergent solution (7.5% [w/v] n-octyl glucopyranoside, 5 mM cardiolipin in 1 mM diethylenetriaminepentaacetic acid [DTPA]). The kinase reaction was initiated by addition of 0.004 units sphingosine kinase in 50 mM HEPES (pH 7.4), 250 mM NaCl, 30 mM $MgCl_2$, 1 µM adenosine triphosphate, and 5 µCi [32P]⍰ adenosine triphosphate. Samples were incubated for 60 minutes at 37°C with shaking (350 rpm), then extracted in 20 µL 1N HCl, 800 µL $CHCl_3$/CH_3OH/1N HCl (100:200:1, v/v/v), 240 µL $CHCl_3$, and 2 M KCl. The lower phase was collected, dried, dissolved in 20 µL of $CHCl_3$:CH_3OH (1:1, v/v), and separated on silica G60 thin layer chromatography plates using $CHCl_3$/CH_3OH/acetic acid/H_2O (90:90:15:5, v/v/v/v) for sphingosine. The thin layer chromatography plates were analyzed using a phosphoimager.

3.9 Statistical Methods

Mean bacterial counts (cfu) were calculated and reported ± standard deviation. Mean bacterial counts from sphingosine-coated, phytosphingosine-coated, and silver-coated ETT segments were compared to vehicle-coated segments and silver-coated segments using Student's t-test in Microsoft Excel for Mac 2011 (IBM, Armonk, NY).

4.0 RESULTS

4.1 Bacterial Adherence to ETTs

ETTs were coated with sphingosine and phytosphingosine in reagent grade hexane and acetone, respectively. Coating tube segments with solvent did not significantly affect the adherence of AB, PA, or MRSA to the surface of the PVC compared to uncoated controls (p=0.78, 0.63, 0.73, respectively). As shown in Figure 1, sphingosine-coated and phytosphingosine-coated tube segments provided significant reductions in AB, PA, and MRSA adherence. Isolation of bacteria adherent to sphingosine-coated tube segments revealed counts of 1.15×10^5 cfu/1-cm segment, 4.05×10^5 cfu/1-cm segment, and 6.67×10^4 cfu/1-cm segment compared to hexane-coated segment counts of 1.82×10^7 cfu/1-cm segment, 1.22×10^7 cfu/1-cm segment, and 1.57×10^6 cfu/1-cm segment of AB, PA, and MRSA, respectively. Isolation of bacteria adherent to phytosphingosine-coated segments revealed 4.18×10^6 cfu/1-cm segment, 2.58×10^6 cfu/1-cm segment, and 6.38×10^5 cfu/1-cm segment compared to acetone-coated segments, 4.22×10^8 cfu/1-cm segment, 2.50×10^7 cfu/1-cm segment, and 1.03×10^8 cfu/1-cm segment, respectively.

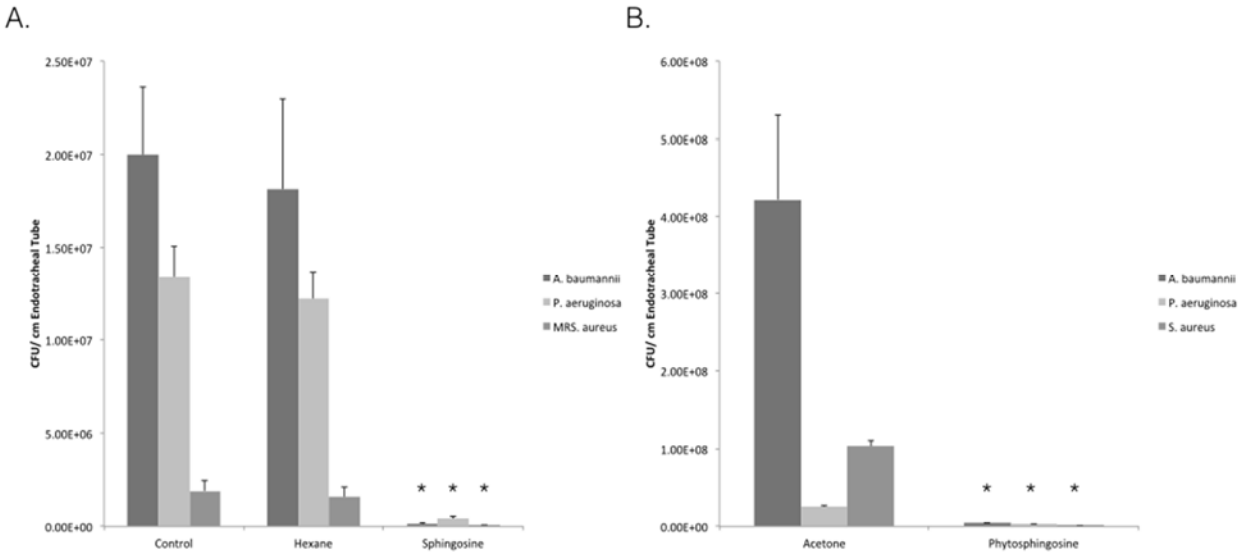

Figure 1. Antimicrobial efficacy of sphingosine- and phytosphingosine-coated ETTs after complete immersion in bacterial suspension. *In vitro bacterial adherence of AB, PA, and SA to (A) uncoated vs. vehicle (hexane)-coated vs. sphingosine-coated and (B) vehicle (acetone)-coated vs. phytosphingosine-coated 1-cm-long segments of standard PVC ETTs. Each tube segment was placed into bacterial suspension containing 1k cfu in growth media and incubated for 12 hours at 37°C. Tube segments were then rinsed in H/S and sonicated to release adherent bacteria. (A) Hexane-coated segments did not significantly reduce the number of adherent bacteria. Sphingosine-coated segments prevented 99.4% (p<0.005), 97% (p<0.005), and 97% (p=0.05) bacterial adherence of AB (n=6), PA (n=6), and SA (n=3), respectively. (B) Phytosphingosine-coated segments prevented 99.0% (p=0.009), 90% (p<0.005), and 99.4% (p<0.005) bacterial adherence of AB (n=5), PA (n=5), and SA (n=5), respectively.*

4.2 Coating Optimization

Quantifying bacteria adherent to surfaces after 24-hour incubation immersed in bacterial suspension reaching greater than 107 cfu/mL (data not shown) is not the most clinically relevant model of biofilm formation on ETTs. ETTs in vivo are inoculated with bacteria, which adhere to the surface prior to biofilm formation. The source of the bacterial inoculant (i.e., oral secretions, gastric reflux, inhaled droplets, etc.) has been reviewed multiple times [17]. Regardless of the mechanism, bacteria adherent to the surface of an ETT are likely subjected to an environment exposed to humidified air. Thus, to simulate this condition, we aimed to modify our antimicrobial assay. Additionally, bacteria adherent to ETTs were difficult to image via microscopy secondary to the curved nature of the tube. Thus, we decided to coat flat plastic coverslips as a surrogate for PVC ETTs.

The antimicrobial assay we used is a variation of the international standard for measurement of antibacterial activity on plastics and other non-porous surfaces, ISO 22196. Briefly, 10,000 cfu of AB, PA, or MRSA in 10 μL TSB bacterial growth media was placed on the coated coverslips, covered with a 2-cm x 3-cm piece of LDPE plastic film, incubated at 37°C for 24 hours, and rinsed. To simulate the environment of an ETT in vivo, we then suspended the coverslips in air and incubated them at 37°C, 100% humidity for 12 hours.

The coverslips were coated with phytosphingosine as described in the Methods section, resulting in a 2.4-cm x 4-cm area of coated plastic that was then subjected to the variation of ISO 22196. As shown in Figure 2A, we found phytosphingosine-coated plastic significantly reduced AB, PA, and MRSA adherence. Isolation of AB, PA, and MRSA adherent to phytosphingosine-coated plastic revealed bacterial counts of 1.74×10^5 cfu, 3.43×10^4 cfu, and 2.75×10^5 cfu compared to ethanol-coated counts of 4.68×10^6 cfu, 4.59×10^6 cfu, and 4.01×10^6 cfu, respectively.

To assess the durability of the phytosphingosine coating against bacteria, we performed the above protocol with the addition of a second and third inoculation of 10,000 cfu of AB, PA, or MRSA in 10 μL TSB bacterial growth media at the 24-hour and 48-hour time points. After 72 hours, rinsing, dry incubation, and sonication were performed. As shown in Figure 2B, we found the anti-adherent properties of phytosphingosine-coated plastic were durable at least up to 72 hours. Isolation of adherent AB, PA, and MRSA to phytosphingosine-coated plastic revealed counts of 1.45×10^5 cfu, 4.33×10^5 cfu, and 2.62×10^4 cfu compared to ethanol-coated counts of 2.17×10^6 cfu, 7.33×10^6 cfu, 9.37×10^6 cfu, respectively. Figure 3 shows the results of standard light microscopy to visualize the adherent bacteria.

4.3 Bacterial Adherence to Optimized ETT Coating

After optimizing our coating process utilizing plastic coverslips as a surrogate for PVC ETT segments, we repeated our antimicrobial assay with the following modification: instead of utilizing a small volume of bacterial suspension and covering with a plastic film, we incubated the ETT segments by complete immersion followed by rinsing and suspension in air at 37°C, 100% humidity for 24 hours.

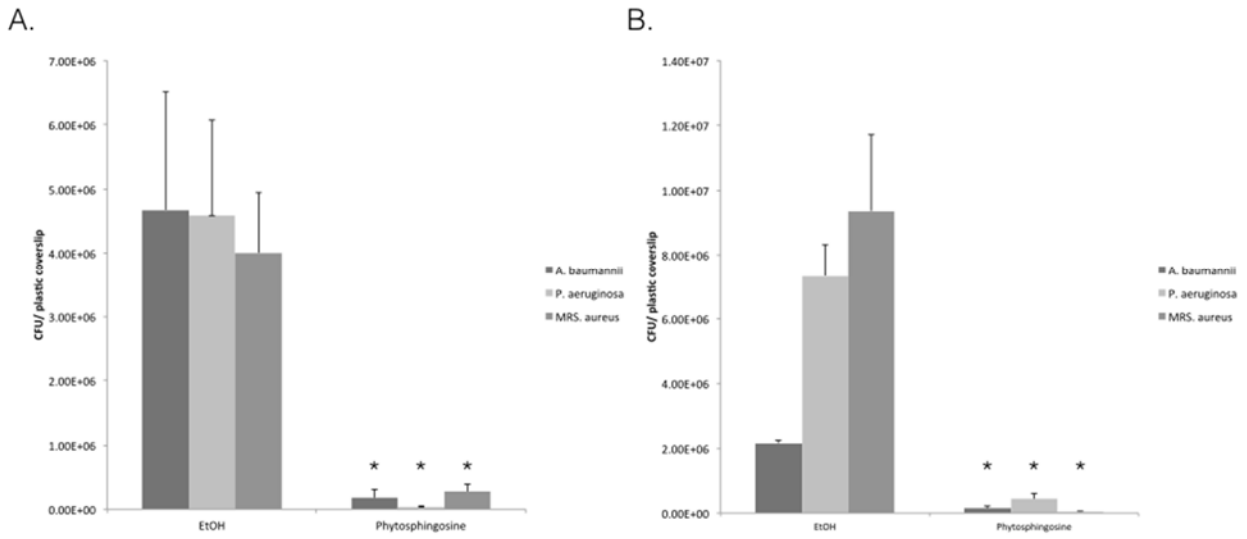

Figure 2. Antimicrobial efficacy of phytosphingosine-coated plastic. *In vitro bacterial adherence of AB, PA, and MRSA to vehicle (ethanol)-coated vs. phytosphingosine-coated plastic coverslips. (A) Bacterial suspension containing 10,000 cfu in growth media was pipetted onto plastic coverslips, covered with plastic film, and incubated for 24 hours at 37°C. Coverslips were rinsed with H/S, incubated for 12 hours, and adherent bacteria were released from the surface via sonication and plated. Phytosphingosine-coated coverslips prevented 96% (p=0.02), 99% (p=0.006), and 93% (p<0.005) bacterial adherence of AB (n=20), PA (n=15), and MRSA (n=20), respectively. (B) Bacterial suspension containing 10,000 cfu in growth media was pipetted onto plastic coverslips, covered with plastic film, and incubated for 24 hours. Additional suspensions of 10,000 cfu were pipetted onto the coverslips after 24 hours and 48 hours. At 72 hours, coverslips were rinsed in H/S, incubated for 12 hours, and adherent bacteria were released via sanitation and plated. Phytosphingosine-coated coverslips prevented 93% (p=0.005), 94% (p=0.005), and 99% (p=0.03) bacterial adherence of AB (n=3), PA (n=3), and MRSA (n=3), respectively.*

As shown in Figure 4, sphingosine-coated and phytosphingosine-coated ETT segments provided significant reduction in AB and SA adherence, and phytosphingosine-coated ETT segments provided significant reduction in PA adherence. Isolation of AB, PA, and SA to sphingosine-coated and phytosphingosine-coated segments revealed counts of 3.87×10^5, 2.23×10^6, and 2.28×10^5 cfu and 5.40×10^3, 3.48×10^4, and 4.06×10^3 cfu, respectively, compared to ethanol-coated segments, which had counts of 2.90×10^7, 9.03×10^6, and 6.72×10^6 cfu, respectively. There were no statistically significant differences between sphingosine-coated and phytosphingosine-coated segments compared to silver, which revealed AB, PA, and SA counts of 2.14×10^6, 1.00×10^3, and 1.38×10^7 cfu, respectively. However phytosphingosine-coated segments showed a trend toward less AB (99.8%, p=0.15) and SA (99.97%, p=0.16) adherence compared to silver, and silver showed a trend toward less PA (97.1%, p=0.05) compared to phytosphingosine.

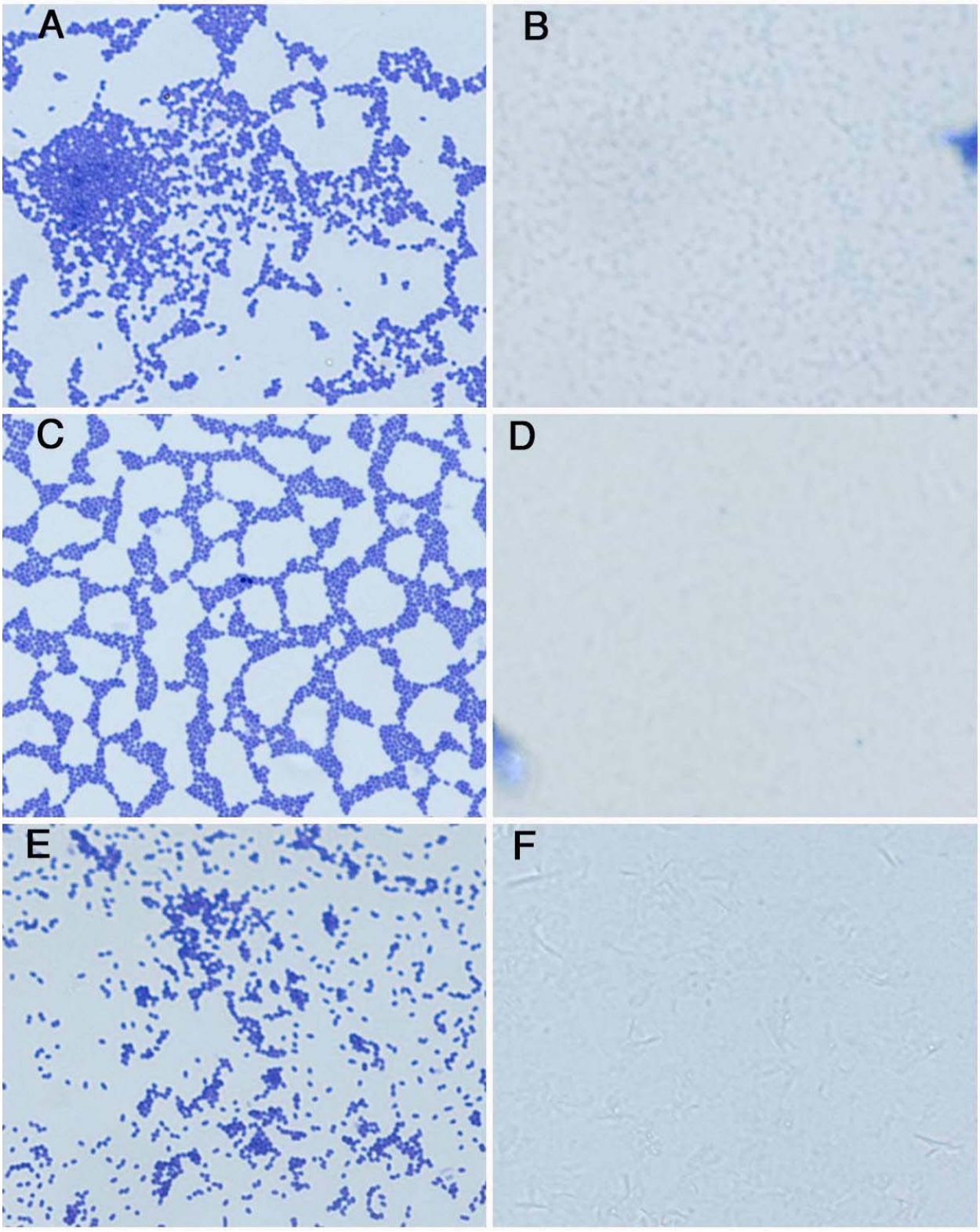

Figure 3. In vitro bacterial adherence of AB, PA, and MRSA to vehicle (ethanol)-coated (A, C, E) compared to phytosphingosine-coated (B, D, F) plastic coverslips. *Adherent bacteria were not isolated via sonication, but rather heat fixed to the plastic slide, stained with crystal violet, and mounted on glass slides (section 3.4).*

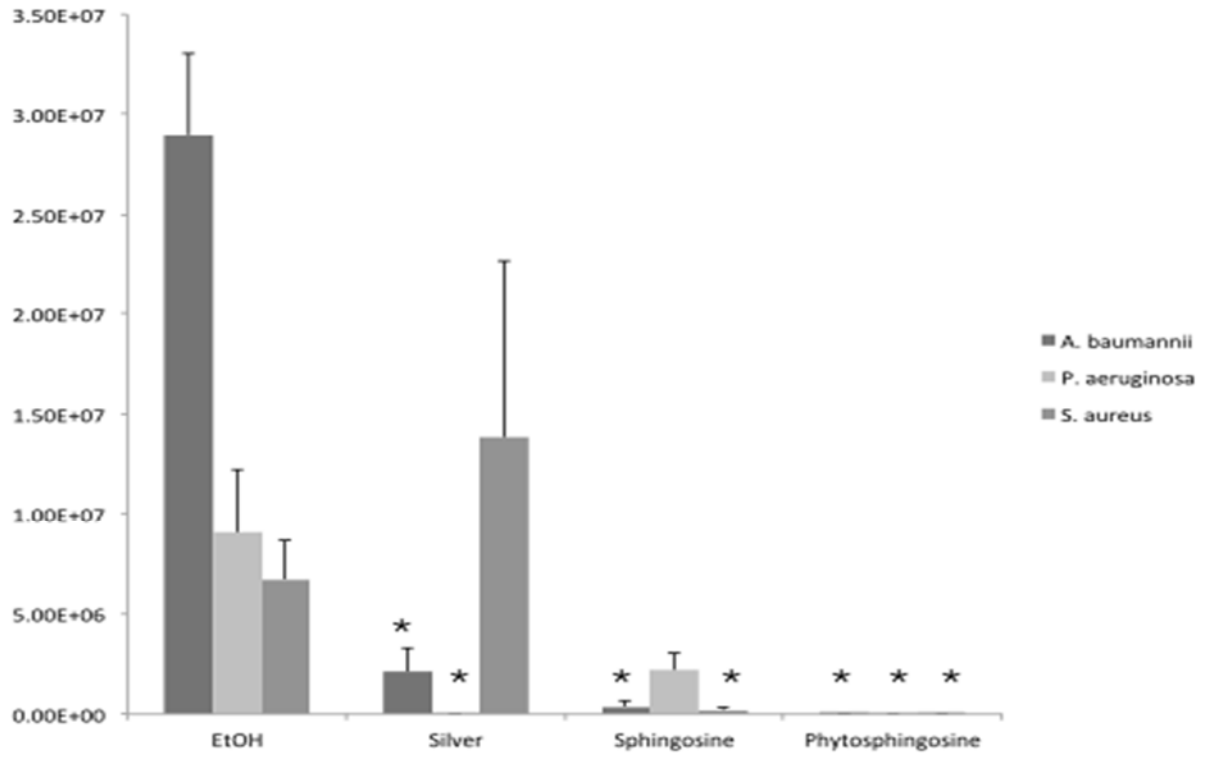

Figure 4. Antimicrobial efficacy of silver-coated (Bard), sphingosine-coated, and phytosphingosine-coated ETTs after immersion and subsequent air incubation at 37°C, 100% humidity. *In vitro bacterial adherence of AB, PA, and SA to vehicle (ethanol)-coated, commercially available silver-coated, sphingosine-coated, and phytosphingosine-coated 1-cm-long segments of 8.0 standard PVC ETTs. Each tube segment was incubated in bacterial suspension containing 1k cfu in TSB growth media, incubated at 37°C for 24 hours, rinsed in 50 mL H/S, incubated suspended in air at 37°C for 24 hours, sonicated for 15 minutes to release adherent bacteria, and plated. Silver-coated segments prevented 93% ($p<0.005$) and 99.99% ($p=0.05$) bacterial adherence of AB and PA, respectively, but did not prevent adherence of SA compared to vehicle-coated controls. Sphingosine-coated segments prevented 99% ($p<0.005$), 75% (not statistically significant, $p=0.13$), and 97% ($p=0.008$) and phytosphingosine-coated segments prevented 99.98% ($p<0.005$), 99.6% ($p=0.05$), and 99.94% ($p=0.006$) bacterial adherence of AB ($n=4$), PA ($n=4$), and SA ($n=8$), respectively.*

4.4 Characterization of Sphingolipid-Coated ETTs

Sphingosine and phytosphingosine are molecules found on various biological membranes of living organisms. They are also classified as nonionic biosurfactants. Adsorption of surfactants onto solid surfaces in aqueous solutions is a well-studied process. Multiple mathematical models have been developed to characterize this process (i.e., Langmuir isotherms). These models describe a process by which a monolayer (or bilayer) of surfactant molecules adsorbs onto solid surfaces. Any attempt at increasing the aqueous concentration of the surfactant to increase the adsorption is limited by the critical micelle concentration of the surfactant. Therefore, adsorption of coating thicker than a mono- or bilayer is a laborious process (i.e., Langmuir-Blogett films). Our method of evaporative-induced deposition of nonionic surfactants is not limited by the same parameters. As shown in Figure 5, our coating method results in adsorption of three-dimensional surfactant structures with features as large as 20 μm in

diameter. These three-dimensional structures contain large amounts of adsorbed surfactant compared to monolayers (or bilayers) while still <10 μm in thickness.

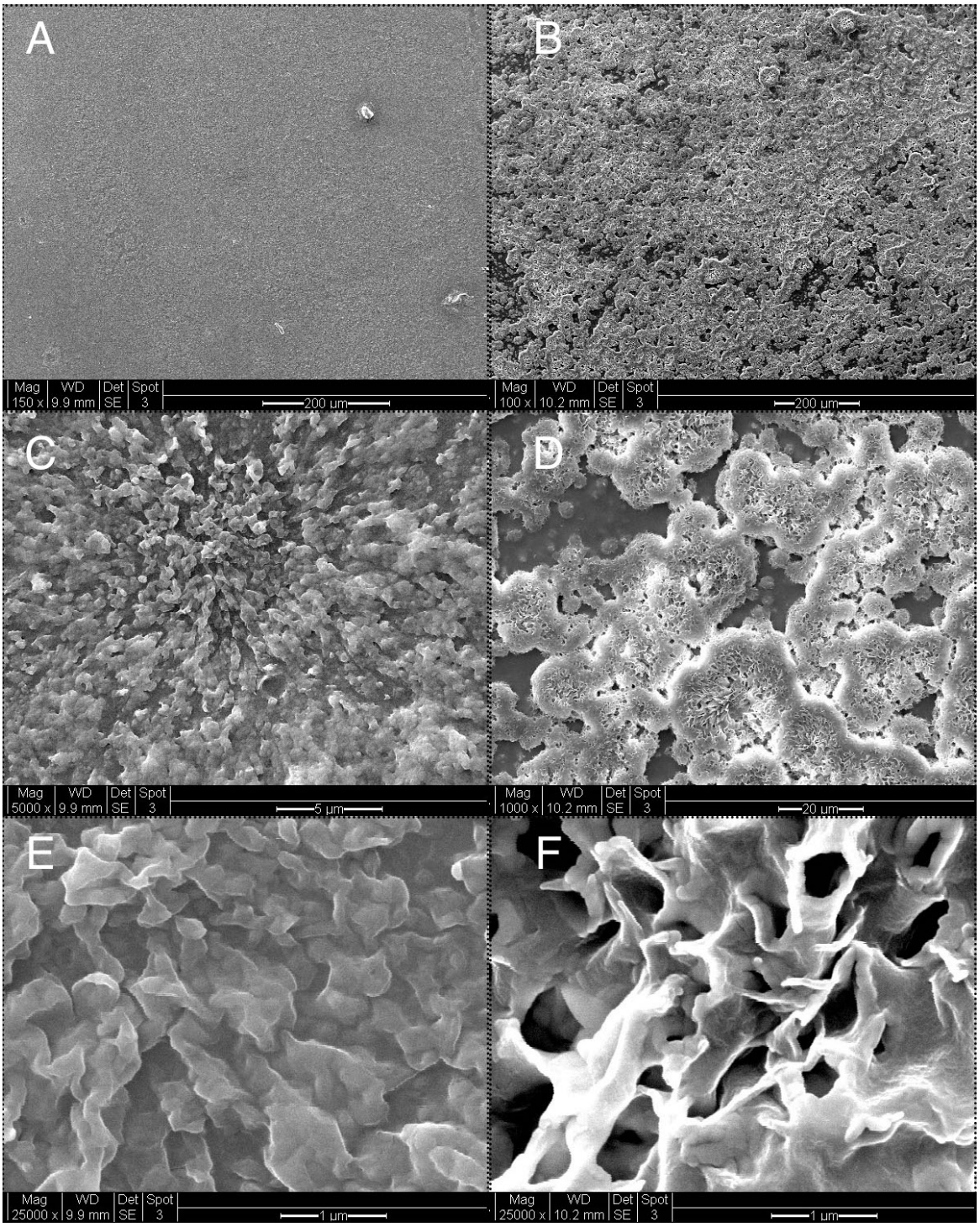

Figure 5. Sphingosine- and phytosphingosine-coated ETTs. *1-cm segments of ETTs were dip coated once in 30 mM sphingosine (A, C, E) or 30 mM phytosphingosine (B, D, F) in 100% ethanol heated to 70°C. Segments were then stained with 0.1% osmium tetroxide, sputter coated with gold/platinum, and imaged with scanning electron microscopy. Images obtained at three different magnifications.*

4.5 Quantification of Sphingosine

To determine the total amount of sphingosine present on the surface of the ETTs after dip coating, we performed mass spectrometry (Figure 6A) and a sphingosine kinase radioactivity assay (Figure 6B). Both methods confirmed a concentration of sphingosine in the nmol to µmol range per cm^2. This is an impressive amount, as sphingosine has been shown to kill bacteria in solution with concentrations of nmol/L to µmol/L.

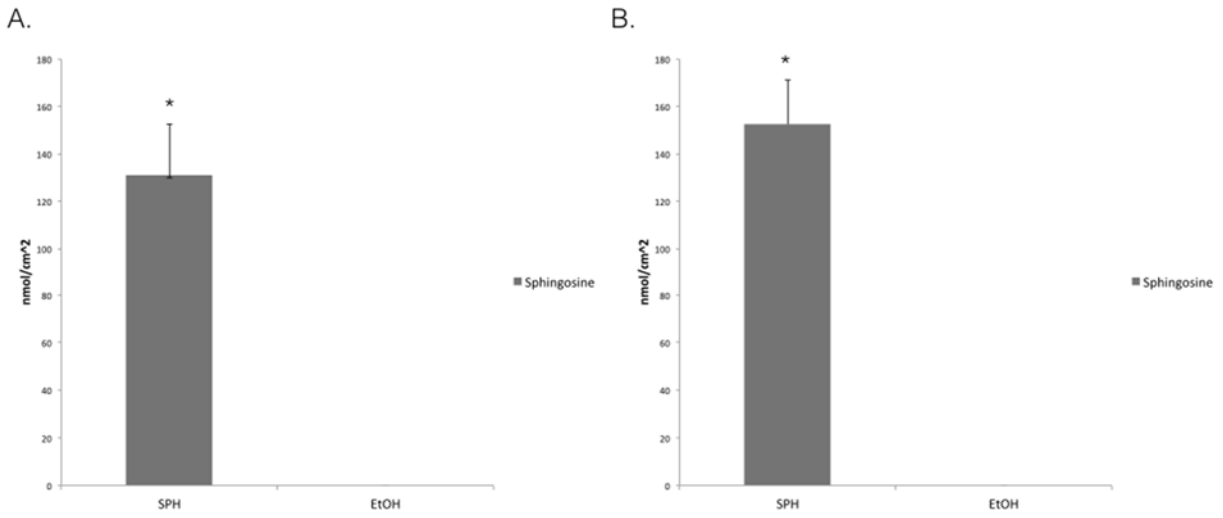

Figure 6. Quantification of sphingosine on the plastic surface. *ETT segments were dip coated in a heated organic sphingosine solution as described above. Sphingosine was either extracted and analyzed by mass spectrometry (A) or sphingosine kinase assay (B). The amount of sphingosine calculated on the surface after dip coating is (A) 131 nmol/cm^2 and (B) 152.5 nmol/cm^2, respectively.*

4.6 Durability of Sphingolipid Coating

Any antimicrobial coating applied to ETTs (or any medical device) must have stability and durability when immersed in biological fluids. We studied the durability of sphingosine coating in urine, blood, and saliva for 7 days quantified by sphingosine kinase assay and in water, H/S, and PBS imaged with electron microscopy. As shown in Figure 7, sphingosine coating was most stable in saliva followed by urine and then blood. The amount of sphingosine quantified after soaking in saliva was nearly double (186%) that of control. This indicates that sphingosine present in the saliva was adherent to the previously sphingosine-coated ETT pieces. Some nonionic surfactants are very soluble in water, and high amounts of adsorbed surfactant become solubilized when placed back in aqueous solutions. However, sphingosine and phytosphingosine are nearly completely insoluble in water, which confers a high degree of stability of sphingosine and phytosphingosine coating when placed into aqueous solutions. Figures 8 and 9 show that the three-dimensional structure of the sphingolipid coatings is stable in aqueous solutions after 12 hours (Figure 8) and 7 days (Figure 9).

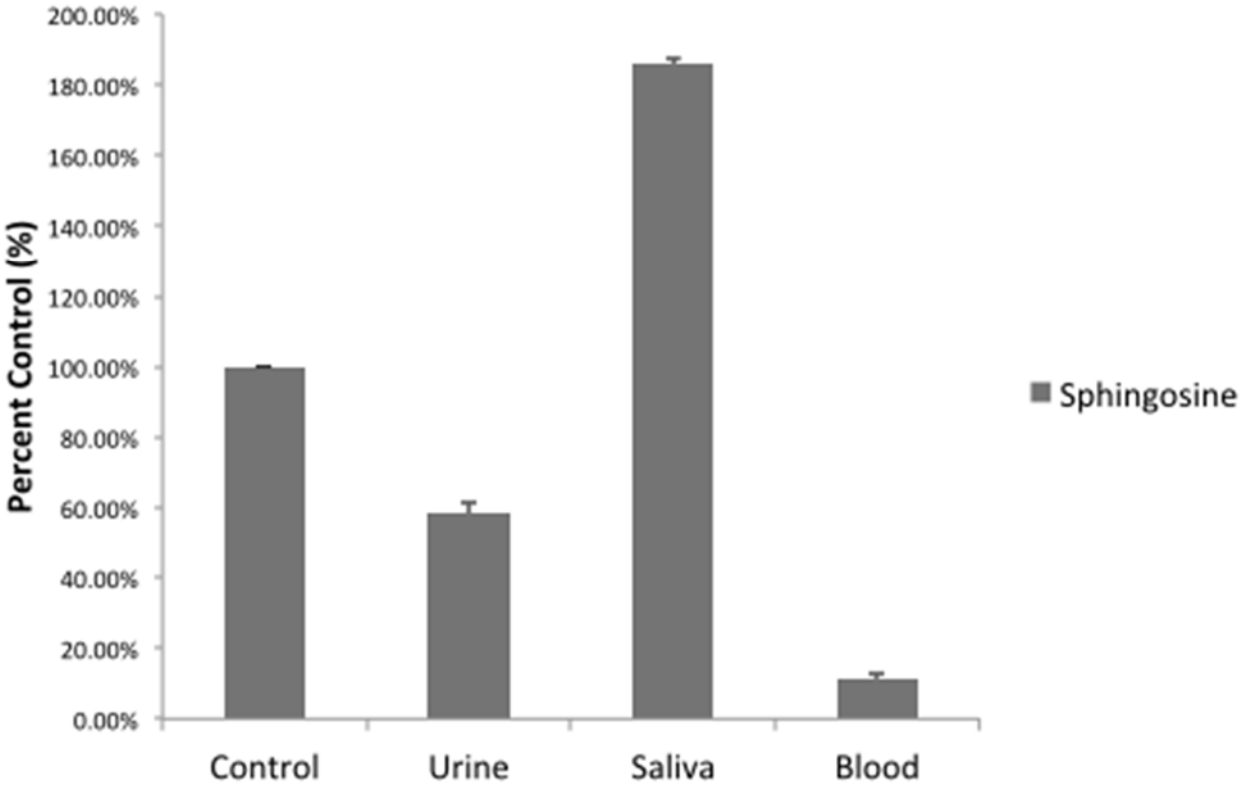

Figure 7. Durability of sphingosine coating in body fluids. *Sphingosine was applied to the ETT by dip coating as described in 3.2. The sphingosine-coated ETT segments were then immersed in urine, saliva, or blood or not immersed (control) and incubated at 37°C for 7 days. Sphingosine was then quantified at the surface using the sphingosine kinase assay described in 3.8. Compared to control, 58% (n=3), 186% (n=3), and 11% (n=3) sphingosine remained on the surface of the ETT segments when placed in urine, saliva, and blood, respectively.*

5.0 DISCUSSION

Our data show that sphingosine-coated and phytosphingosine-coated ETTs are highly efficacious at preventing bacterial adherence against three of the most common pathogens, AB, PA, and SA, associated with VAP when compared with standard plasticized PVC ETTs. Additionally, our data indicate our sphingolipid-coated tubes are equally efficacious as silver-coated ETTs.

VAP continues to be a major cause of morbidity and mortality in critically ill patients. While prompt diagnosis and effective treatment with standard antibiotic regimens are important in mitigating the detrimental effects of VAP, development and implementation of more effective prevention strategies will decrease the incidence and likely provide a greater reduction in morbidity and mortality. Low cost strategies such as semi-recumbent positioning, chlorhexidine oral care, and subglottic suctioning have all been shown to reduce rates of VAP, and studies have shown they have been successfully implemented in community systems [18]. Silver-coated ETTs have also been shown to reduce rates of VAP, but have not shown widespread implementation, possibly secondary to the large cost associated with silver-coated tubes (~$100 per tube when purchased for this study).

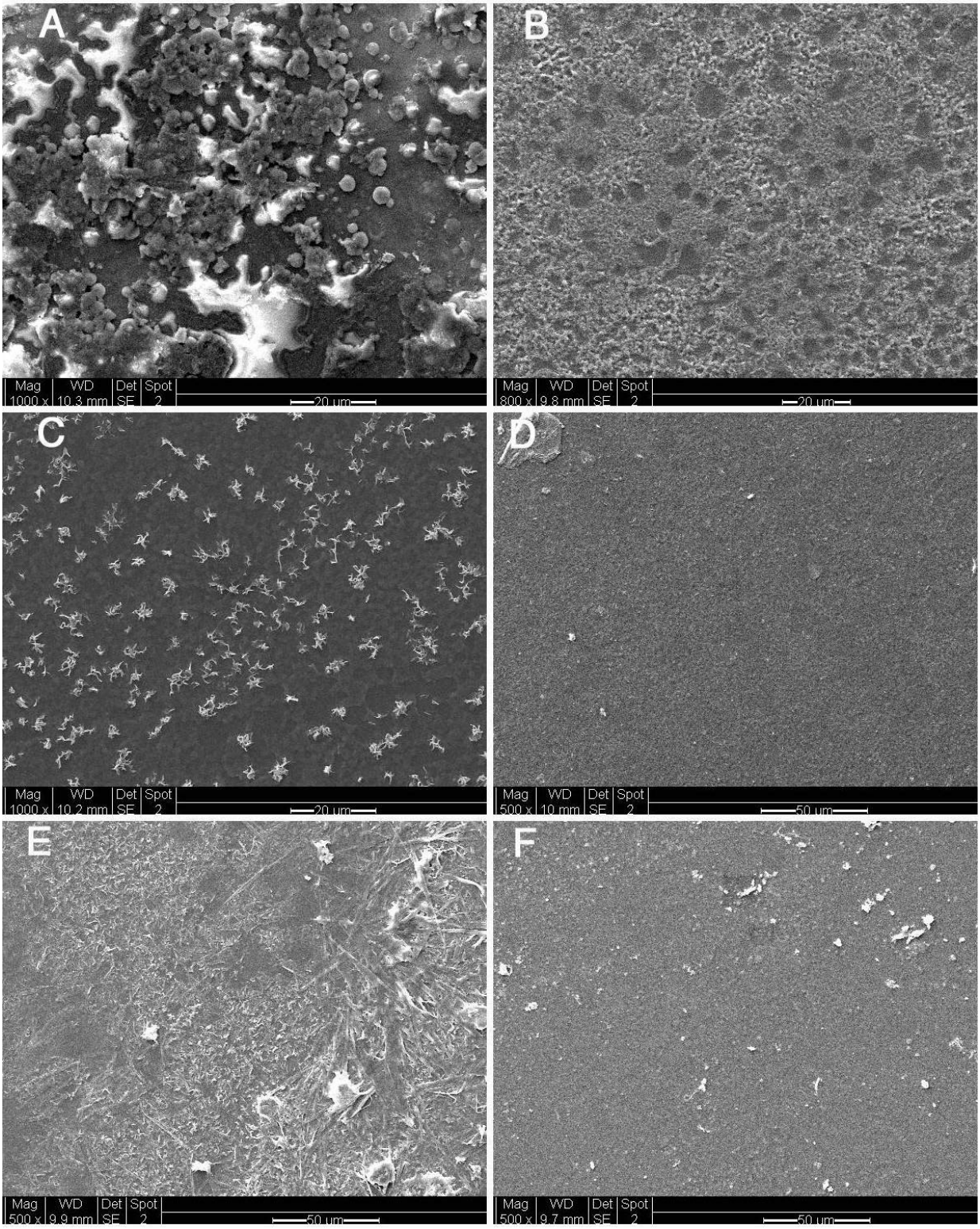

Figure 8. Phytosphingosine- and sphingosine-coated PVC ETT 12-hour aqueous immersion. *1-cm segments of ETTs were dip coated once in 75 mM phytosphingosine (A, C, E) or 90 mM sphingosine (B, D, F) in 100% ethanol heated to 70°C. Segments were unsoaked (A, B), soaked for 12 hours in H_2O [water] (C, D), or soaked for 12 hours in H/S (E, F). Segments were then stained with 0.1% osmium tetroxide, sputter coated with gold/platinum, and imaged with scanning electron microscopy.*

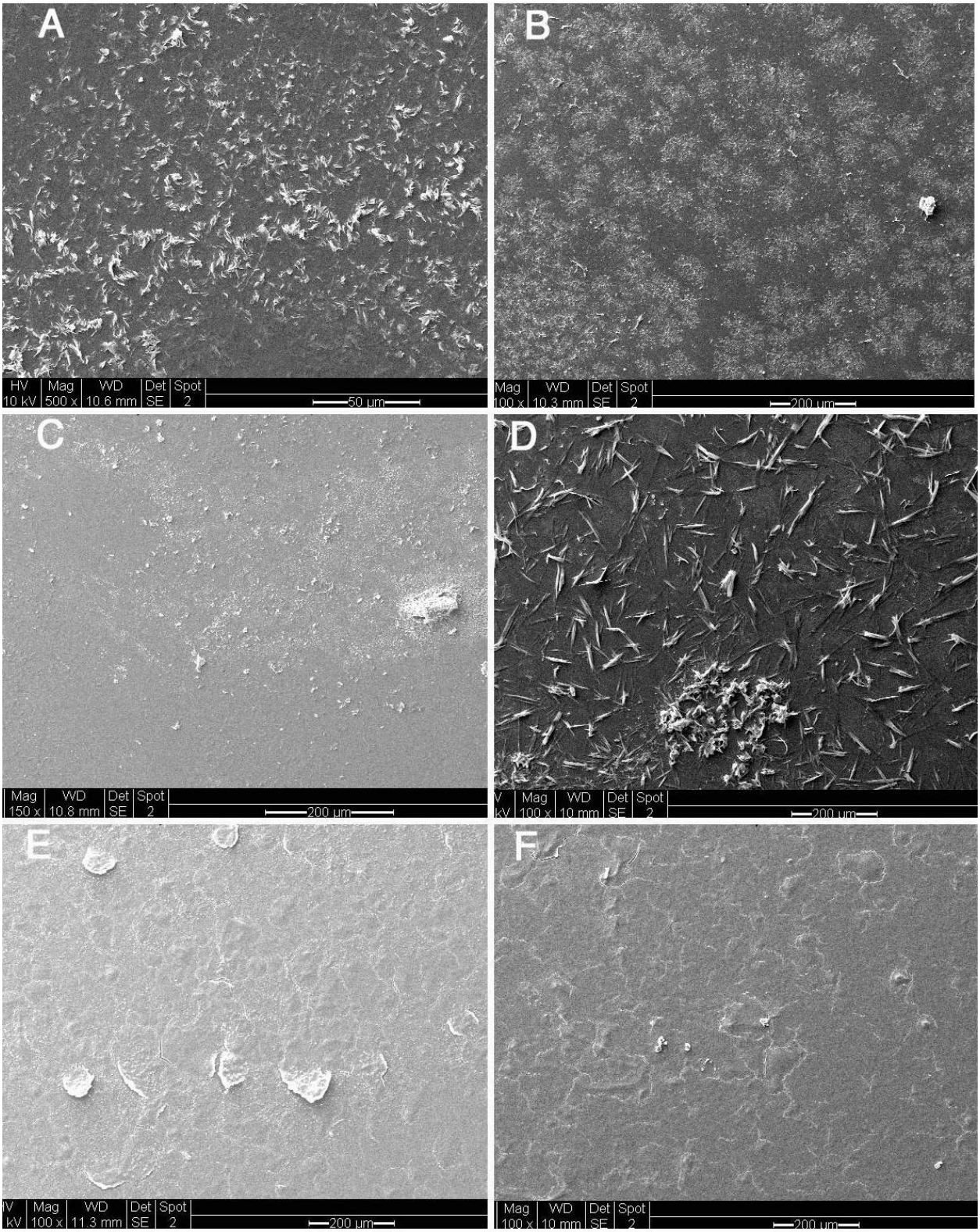

Figure 9. Phytosphingosine- and sphingosine-coated PVC ETT 7-day aqueous immersion. *1-cm segments of ETTs were dip coated once in 75 mM phytosphingosine (A, C, E) or 90 mM sphingosine (B, D, F) in 100% ethanol heated to 70°C. Segments were soaked for 7 days in H_2O (A, B), H/S (C, D), or phosphate buffered saline (PBS) (E, F). Segments were then stained with 0.1% osmium tetroxide, sputter coated with gold/platinum, and imaged by scanning electron microscopy.*

The cause of VAP is likely multifactorial, but the presence of a biofilm that develops after only 24 hours of tracheal intubation has been identified a likely source of infection [19,20]. A safe antimicrobial coating that can prevent bacterial adherence, the first step in biofilm formation, and can be applied easily and for a low cost has the potential to affect meaningful change in prevention of VAP. Sphingosine is a sphingolipid found in the membranes of most eukaryotic cells. Administration via inhalation was shown not only to reduce rates of pneumonia in susceptible mice, but also did not result in any observable toxicity [14]. Sphingosine and phytosphingosine have been shown to have antimicrobial activity against both Gram-positive and Gram-negative bacteria. Additionally, sphingosine's presence in the skin, oral mucosa, and respiratory mucosa suggests sphingosine is a part of mammalian innate immunity. Thus, it is likely that sphingosine's antimicrobial mechanism is not prone to development of bacterial resistance.

Our evaporative-induced deposition of sphingosine via dip coating is a simple and cheap way to apply a biologically significant amount of sphingosine on the surface of ETTs. Our method only requires a single dip and thus uses a very small volume of sphingolipid solution. We calculated the raw materials cost of coating a single ETT to be less than $0.01.

6.0 CONCLUSIONS

Sphingosine and phytosphingosine coating effectively prevent adherence of *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* to the surface of endotracheal tubes with an effectiveness comparable to silver coating. Sphingosine and phytosphingosine were shown to have an antimicrobial durability of at least 3 days and a coating durability in aqueous solutions and saliva of at least 7 days. Future in vitro and animal studies are necessary to establish the safety of sphingolipid coatings, and future randomized clinical trials will be necessary to determine sphingosine or phytosphingosine's ability to provide a cost-effective preventative strategy to reduce rates of ventilator-associated pneumonia.

7.0 REFERENCES

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LIST OF ABBREVIATIONS AND ACRONYMS

AB	*Acinetobacter baumannii*
cfu	colony forming units
$CHCl_3$	chloroform
CH_3OH	methanol
ETT	endotracheal tube
H/S	HEPES/saline
HCl	hydrochloric acid
HPLC	high performance liquid chromatography
KCl	potassium chloride
LDPE	low density polyethylene
$MgCl_2$	magnesium chloride
MRSA	methicillin-resistant *Staphylococcus aureus*
NaCl	sodium chloride
PA	*Pseudomonas aeruginosa*
PBS	phosphate buffered saline
PVC	polyvinyl chloride
SA	*Staphylococcus aureus*
SEM	scanning electron microscopy
TSB	trypticase soy broth
VAP	ventilator-associated pneumonia