Electron microscopic analysis of biofilm on endotracheal tubes removed from intubated neonates

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OBJECTIVES: **To determine if the phenomenon of biofilm accumulation and associated microbial colonization occurs on the surface of endotracheal tubes in the region of the subglottis in neonates.**

METHODS: **Endotracheal tubes removed from 9 consecutive neonatal patients intubated for more than 12 hours were processed (range, 13 hours to 8 days). A sterile control tube was also processed. For each, the portion of the endotracheal tube that had been in contact with the subglottis was determined using a previously published nomogram. A 1-cm-long cross-sectional segment of the endotracheal tube corresponding to the level of the subglottis was divided into 2 portions for both electron microscopy and aerobic/anaerobic cultures.**

RESULTS: **Two of 9 (22%) luminal surface cultures grew *Staphylococcus* species, 1 (11%) grew normal flora, and 6 (66%) had no growth. Three of 9 (33%) outer-surface cultures grew *Staphylococcus* species, 1 (11%) had gram-negative rods on staining but a sterile culture, and one enterococcal contaminant was found. Electron microscopy revealed that 8 of 9 inner lumen surfaces harbored bacteria and biofilm formation. All outer lumen surfaces had biofilm formation; 6 of 9 had bacterial colonization. There was no obvious difference in the appearance of the inner and outer tube surface accretions. No time-dependent differences were noted except of the longest indwelling tube (8 days).**

CONCLUSION: **This study demonstrates for the first time the presence of biofilm on the outer surface of neonatal endotracheal tubes. The data suggest that the presence of bacteria and/or biofilm does not correlate with other traditional indicators of microbial colonization.** (Otolaryngol Head Neck Surg 2004;130:407-14.)

In neonates, acquired subglottic stenosis is a known sequela of prolonged intubation. The presumed mechanism of this complication is pressure ischemia, necrosis, and breakdown of the subglottic mucosa. Local invasion of bacteria follows, which leads to subglottic chondritis, and appears to be a step in the development of this condition.[1] It is not unreasonable to postulate that a protective layer of biofilm over the endotracheal tube in the region of the subglottis may enhance the likelihood of chondritis, suggesting a possible role for biofilm in the genesis of subglottic stenosis.

To date, only two prior studies have investigated endotracheal biofilm formation in neonates. One study reported on 29 tubes,[2] while the other reported on 14 such specimens.[3] All tubes in both studies were coated with luminal biofilm, with the material appearing as early as 11 hours after intubation.[2] Similar to adult studies, bacterial structures were frequently found within the biofilm layer.[2,3] However, no study in either adults or neonates has searched for the presence of biofilm on the external surface of endotracheal tubes, which is the surface of the tube that is in direct contact with the patient's upper airway mucosa.

In preliminary work performed at our institution,[4] the external surfaces of 33 endotracheal tubes were swabbed for culture after removal from patients in the neonatal intensive care unit. Cultures were taken from areas of the tubes corresponding to the regions of the distal trachea, subglottis, and pharynx. Polymicrobial flora was found in approximately 50% of specimens, distributed evenly between sites. Colonization was shown to be time dependent, with a statistically significant increase in the incidence of a positive culture after 4 days of intubation. A possible role for biofilm was suggested, but electron micros-

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copy was not performed to confirm the presence of such a layer.

In the present study, neonatal endotracheal tubes were subjected to both culture processing and electron microscopy. Unlike most previous studies, this investigation attempted to identify biofilm on the luminal and the external tube surface, and attempted to correlate the presence of microbial structures seen on electron microscopy with standard culture isolates.

PATIENTS AND METHODS

Patients

All intubated newborns in the neonatal intensive care unit were eligible for inclusion in this study, with the exception of neonates who had undergone previous airway surgery or those in whom the endotracheal tube could not be immediately processed after extubation. Institutional Review Board approval was obtained for this study.

Endotracheal tubes (cuffless, polyvinyl tubes manufactured by Mallinckrodt, Inc., St. Louis, MO) from 9 consecutive extubations (Table 1) and 1 control sterile tube were processed as described below. Clinical data for each patient included: gestational age at birth, admitting diagnosis, birth weight, crown-rump length (CRL), duration of intubation, previous intubation history, antibiotic and H2-blocker therapy, and the distance of endotracheal tube tip to carina on last chest radiograph.

Calculation of Subglottic Point

For each subject, the portion of the endotracheal tube that had been in contact with the subglottis was determined using a nomogram devised by Rotschild et al.[5] The last chest radiograph for each neonate was examined and the distance from the tip of the endotracheal tube to the carina was measured. This distance (C) was not corrected for radiograph magnification, as previous experience with this technique has demonstrated negligible magnification in this setting.[4] Then, the length of the trachea was determined by correlation with the patient's crown-rump length (CRL) using the following formula: $L = (0.708 + 0.106)$ CRL (cm).

The distance from the endotracheal tube tip to the subglottis (S) was then determined by the following formula: $S = L - C - 0.3$ (cm).

The factor 0.3 cm was added to ensure that the sampled section was not above the level of the vocal folds.

Collection of Endotracheal Tubes

A sterile work area was prepared next to each infant at the time of extubation. Included on the field were a ruler, a knife, culture swabs and tubes, and a vial filled with 3% glutaraldehyde. The above formulas were used to calculate the level of the subglottis on the chest radiograph and this point was marked on the ruler. A 1-cm cross-sectional segment of the endotracheal tube corresponding to the subglottis was cut with the sterile knife (0.5 cm below and 0.5 cm above the mark). The 1-cm segment was then divided cross-sectionally into two 0.5-cm segments. Swabs of the external surface and then (separately) the inner surface of the first 0.5-cm segment were sent for bacterial (aerobic/anaerobic) and fungal cultures. The remaining 0.5-cm segment was divided longitudinally into two equal segments, both of which were placed in a vial filled with 3% glutaraldehyde and sent for scanning electron microscopy (EM) of both the outer and inner surfaces of the endotracheal tube.

Electron Microscopy

After collection, specimens were fixed in 3% glutaraldehyde in a 0.2 M sodium cacodylate buffer at pH 7.4, followed by treatment with 1% osmium tetroxide for 1 hour. The material was then dehydrated in graded ethanol steps: 50%, 70%, 95%, 100%, and then critical point dried with liquid CO_2 and lightly coated with coated gold-palladium. The specimens were observed with a Hitachi S530 scanning electron microscope (SEM).

RESULTS

There were 9 consecutive extubations from 8 neonates. One unused tube was fixed and studied in the same manner and served as a control. The duration of intubation ranged from 13 hours to 8 days (mean 46 hours, median 72 hours). Admission diagnoses included: sepsis post–corrective cardiac surgery (3), hyaline membrane disease (2), anemia (1), prematurity (1), postoperative sepsis (1), and respiratory failure (1).

Table 1. Patient data

	Diagnosis	Days of Intubation	Previous Intubation	IV antibiotics	H2 blockers	Gestational Age
1	Coarctation of aorta, aortic thrombectomy	3	(+) 4 days	Ancef	Zantac	39 4/7 week
2	Fetal-maternal transfusion	5	No	Ampicillin/Gentamicin	None	33 weeks
3	HMD	13 hours	No	IM Penicillin ×1 dose	None	33 weeks
4	Gastroschisis, line sepsis, respiratory failure	5	For surgery 1 month prior	Nafcillin, Gentamicin, Ampicillin, Flagyl	Carafate	30 weeks
5	Respiratory distress	14 hours	No	Ampicillin	None	32 weeks
6	HMD	8	No	Ampicillin, Gentamicin	None	28 weeks
7	Prematurity	1	No	IM Penicillin ×1 dose	None	26 1/7 weeks
8	Pulmonary atresia, PDA, CHF, sepsis	3 (DOL#17–19)	No	Cefotaxime, Nafcillin	None	32 weeks
9	Same patient as #8	4 (DOL#23–26)	Yes	Cefotaxime, Nafcillin, Amphotericin	None	32 weeks

DOL = day of life, HMD = hyaline membrane disease, PDA = patent ductus arteriosus, CHF = congestive heart failure

Table 2. Culture results of inner and outer surfaces of the endotracheal tubes

	Inner Lumen Bacterial	Inner Lumen Fungus	Outer Surface Bacterial	Outer Surface Fungus
1	Staph epidermidis	(−)	Staph epidermidis	(−)
2	(−)	(−)	(−)	(−)
3	(−)	(−)	(−)	(−)
4	(−)	(−)	(−) culture Gram-negative rods on gram stain	(−)
5	(−)	(−)	Coagulase-negative Staph (Probable contaminant)	(−)
6	Coagulase-negative Staph, light growth	Penicillium contaminant	Coagulase-negative Staph	(−)
7	(−)	(−)	(−)	(−)
8	(−)	(−)	Enterococcus faecium (? contaminant)	(−)
9	Normal flora	(−)	Normal flora	(−)

Two of 9 (22%) cultures of the luminal (inner) surface of the endotracheal tube grew *Staphylococcus species*, one inner tube culture grew normal flora and 6 of nine 9 (66%) of the tubes grew no organisms. The outer endotracheal tube surface cultures grew *Staphylococcus* species in 3 of 9 (33%) cases, 1 (11%) revealed Gram-negative rods on gram stain but no organism grew on culture, and 1 culture grew a likely contaminant of *Enterococcus faecium* from the broth culture only. Workup of this last patient revealed no *Enterococcus* in subsequent blood or urine cultures. There was growth of normal flora in 1 of 9 (11%) tubes and no growth in 3 of 9 specimens (44%). All of the fungal cultures of the inner and outer endotracheal tube surface were negative (Table 2).

The 10 hemi-tube segments that were stored in 3% glutaraldehyde were processed for scanning electron microscopy as described. The inner and outer tube surfaces were viewed and analyzed by the first author (KBZ) and an EM technician. Independently, to blind the readings, a senior pa-

Table 3. Scanning electron microscopy results

	Inner surface	Outer surface
1	*No bacteria, no §biofilm	(+) biofilm, possible organisms
2	(+) bacteria, (+) biofilm	No bacteria
3	(+) bacteria, (+) biofilm	Possible bacteria, (+) biofilm
4	(+) bacteria, (+) biofilm	(+) bacteria, (+) biofilm
5	(+) bacteria, (+) biofilm	(+) bacteria, (+) biofilm
6	(++) bacteria, (++) biofilm	(++) bacteria, (++) biofilm
7	(+) bacteria, (+) biofilm	(+) bacteria, (+) biofilm (less than inner surface)
8	(++) bacteria, (+) biofilm	(++) bacteria, (+) biofilm
9	(+) bacteria, (+) biofilm	(+) bacteria, (+) biofilm
10 CONTROL	No bacteria, No biofilm	No bacteria, No biofilm

*No bacteria, Presence of bacteria colonies (+), Significant bacterial trapping in biofilm (++)
§No biofilm (−), Presence of biofilm layer (+), Significant biofilm matrix (++)

thologist reviewed all of the photomicrographs. The presence of bacteria, the extent of biofilm formation, and any unusual features were recorded. Table 3 summarizes the results.

Inner Lumen

Bacterial elements were seen on 8 of 9 inner lumen tubes with exuberant growth of bacterial colonies in 2 of the 8 specimens. Biofilm formation was seen on 8 of the 9 specimens, and neither bacteria nor biofilm formation was seen on the control endotracheal tube.

Outer Surface

All of the outer endotracheal tube surfaces obtained after extubation exhibited biofilm formation. Bacterial colonization was seen in 6 of the 9 specimens; 2 of these had exuberant colonization. There was no biofilm or bacterial colonization of the control/sterile endotracheal tube outer surface (Fig 1).

Comparing the inner and outer surfaces of the endotracheal tubes, there was no significant subjective difference in the appearance of these accretions. Furthermore, there was no time-dependent difference in the amount of film on the endotracheal tube, with the exception of the longest indwelling tube (8 days) (Fig 4). A thin layer of amorphous material was noted as early as 14 hours (Fig 2); a similar pattern of debris was noted in tubes that were indwelling up to 3 days (Fig 3). Interestingly, the outer surface of the endotracheal tube appeared to accumulate less debris than the inner tube surface.

Chi-square analysis of the data revealed no correlation between coccal colonization on electron microscopy and culture results. There was no correlation between duration of intubation and culture positivity; and finally, there was no correlation between the use of antibiotics and the presence of bacterial colonies on electron microscopy or on culture.

CONCLUSIONS

Endotracheal tube bacterial colonization was demonstrated as early as 1967, when Redman and Lockey dipped the distal aspect of endotracheal tubes that had been removed from mechanically ventilated patients into nutrient broth for culture.[6] Most subsequent studies have obtained cultures by swabbing the tube lumens shortly after extubation, with the percentage of tubes colonized with microbes commonly ranging from 50% to 90%.[7-11] While normal upper respiratory flora are the most common isolates, up to nearly half of colonized tubes have yielded potential pathogens such as a variety of Gram-negative bacilli, *Staphylococcus* species, and *Candida albicans*.[3,6,8-12]

The formation of an adherent matrix of bacteria on the surfaces of implanted materials is termed "biofilm." The use of electron microscopy to demonstrate the presence of endotracheal tube luminal biofilm was first reported by Sottile et al in 1986.[9] This and subsequent reports have found luminal biofilm to be present in 84% to 100% of postextubation endotracheal tubes.[2,3,9,11,13] The biofilm, as seen on electron microscopy, is typically described as an amorphous material, or accretion,

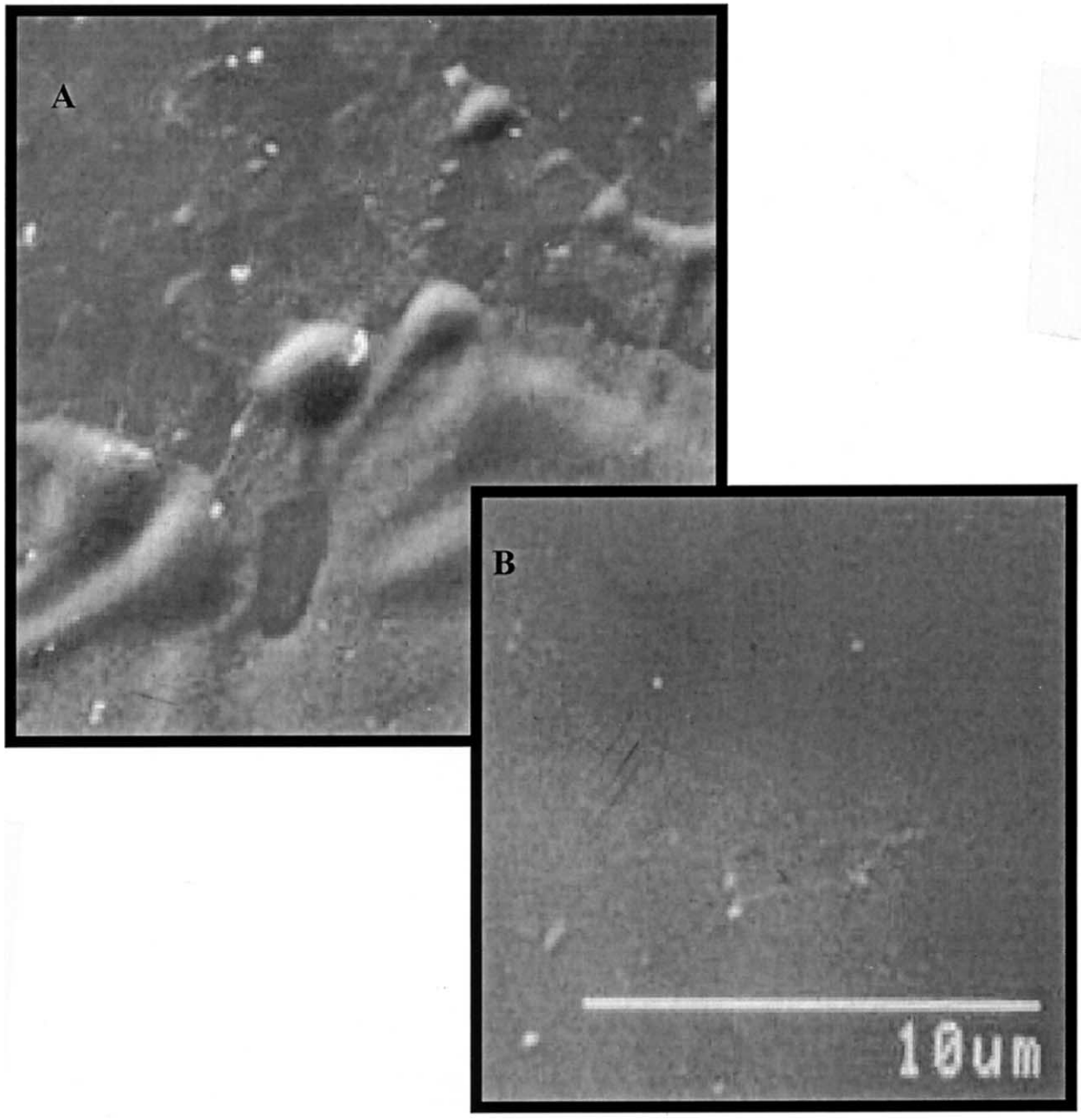

Fig 1. Control, sterile tube. **(A)**: Inner tube lumen. **(B)**: Outer tube surface. Both surfaces had neither biofilm formation nor bacterial colonization; cultures were sterile.

coating the inner tube surface.[2,3,9] The accretion often has deep fissures or cracks in its surface, thought to result from the critical-point drying procedure necessary for scanning electron microscopy.[8,11] Biofilm has been shown to accumulate most regularly in the distal aspect of the tube, while it is less detectable in the more proximal tube regions.[14]

Bacterial structures have been detected within the luminal biofilm on electron microscopy in up to 68% of specimens.[3,9,13] Some authors have reported that these microbes are not found on the surfaces of the biofilm, but rather are visible deep within the cracks and fissures.[8,11] Other authors, however, have described bacterial structures as residing on the surface of the amorphous layer,

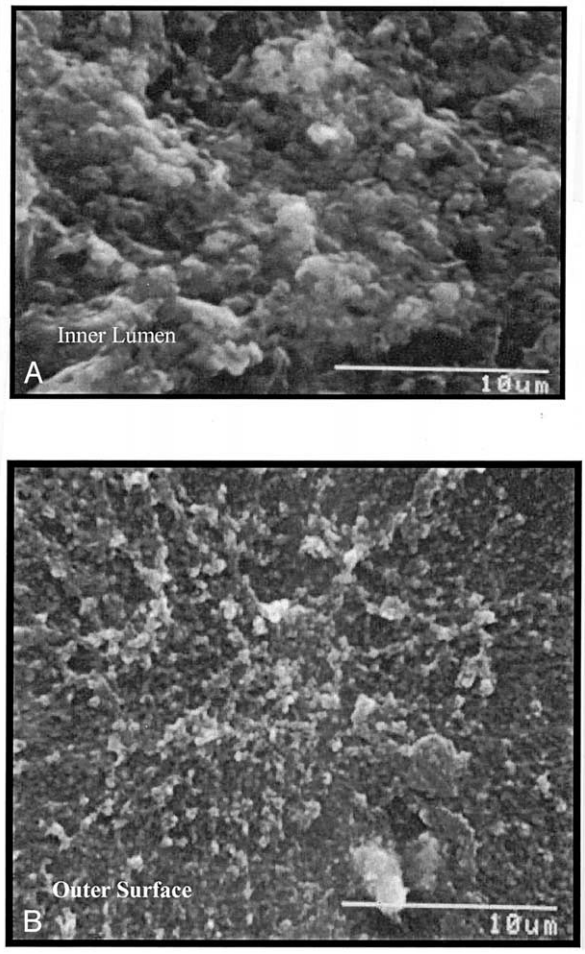

Fig 2. Fourteen hours postintubation. **(A)**: Inner surface of endotracheal tube. Note attached coccal colonies within biofilm layer. Culture of the inner surface of this tube revealed no bacteria. **(B)**: Outer surface of tube. Note presence of bacterial colonies embedded in biofilm. Tracheal tube culture of outer surface grew coagulase-negative staphylococcus.

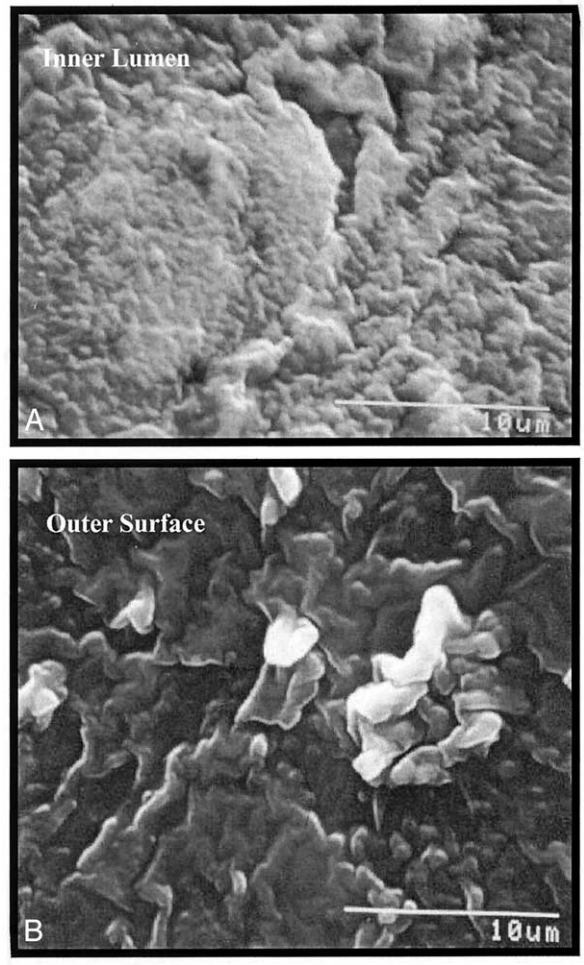

Fig 3. Three days postintubation. **(A)**: Inner tube lumen. Biofilm layer present; no obvious bacterial colonies. **(B)**: Outer surface of tube. Biofilm is clearly visible. No obvious trapped organisms. Cultures of both surfaces yielded coagulase-negative staphylococcus epidermidis.

being separated from the polymer of the tube by the biofilm.[3,13] Direct contact of coccoid structures with the endotracheal tube itself with no intervening biofilm has been described in only one report.[2] In some instances, large clusters of bacterial cells have been demonstrated projecting from the accretion into the tube lumen.[9,11] Some authors have suggested that the likelihood of encountering biofilm microbes on electron microscopy increases with the duration of tube use,[9] although others have found this to not necessarily be the case.[13] The presence of microbial structures on electron microscopy has generally correlated with the presence of positive microbial cultures.[8,9]

The origin of the biofilm found on endotracheal tube surfaces is unknown. It is possible that it may be composed primarily of mucopolysaccharides from respiratory secretions of host origin, rather than polysaccharide glycocalyx of bacterial origin, since luminal accretions have an identical appearance on electron microscopy between colonized and noncolonized tubes.[3] The origin of microbes found within luminal biofilms is also unclear. Since they usually do not represent normal tracheal flora, one must assume that they are in some way aspirated. One possibility is that these represent aspiration of secretions from the oropharynx, a site that is notorious for colonization with Gram-negative bacilli in mechanically ventilated pa-

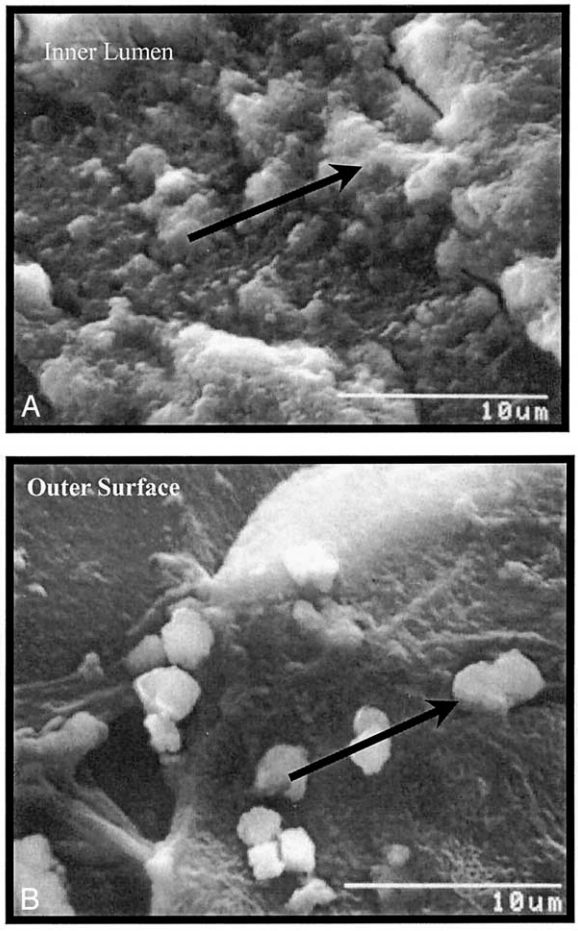

Fig 4. Eight days postintubation. **(A)**: A biofilm layer of inner lumen with obvious, exuberant bacterial colonization. **(B)**: Outer surface of same endotracheal tube with similar findings. Cultures of both surfaces yielded coagulase-negative staphylococcus.

tients.[10,15] Another possible source is from aspiration of gastric or duodenal secretions contaminated with Gram-negative bacilli.[14,15]

In general, microbes that are enmeshed within biofilm are believed to be relatively resistant to the effects of antibiotics.[16,17] Some studies have suggested that the accumulation of bacterial biofilm within the endotracheal tube lumen in mechanically ventilated adults may be a contributing factor in the development of nosocomial pneumonia.[12,15]

This study demonstrates for the first time the presence of biofilm on the outer surface of neonatal endotracheal tubes. The data suggest that the presence of bacteria and/or biofilm on electron microscopy does not correlate with other traditional indicators of microbial colonization. This may imply that the bacteria seen on electron microscopy are not viable pathogens; that the presence of bacteria on electron microscopy may be an early, nonspecific finding for any nonsterile tube; and that the biofilm may indeed be a privileged site where antibiotics have no effect.

Future studies will enable further characterization of the biofilm on the outer and inner lumens of neonatal endotracheal tubes by increasing the power of the study, by scraping the surface of the endotracheal tubes to allow transmission electron microscopic evaluation of the elements. Furthermore, studies will focus on factors that disrupt the biofilm layer to study the effect of biofilm on culture and gram stain results.

Clinical correlation of these studies may in the future suggest new technology such as precoated endotracheal tubes, which could disrupt biofilm formation. Conceivably, this might lessen the impact of nosocomial complications such as pneumonia and/or subglottic stenosis in intubated neonates.

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