

Nasogastric feeding tubes from a neonatal department yield high concentrations of potentially pathogenic bacteria—even 1 d after insertion

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BACKGROUND: Preterm infants are vulnerable to pathogens and at risk of developing necrotizing enterocolitis (NEC) or sepsis. Nasogastric feeding tubes (NG-tubes) might contaminate feeds given through them due to biofilm formation. We wanted to determine if there is a rationale in replacing NG-tubes more often to reduce contamination.

METHODS: We conducted an observational study of used NG-tubes from a tertiary neonatal department. After removal, we flushed a 1-ml saline solution through the tube, determined the density of bacteria by culture, and related it to the duration of use and any probiotic administration through the tube.

RESULTS: Out of the 94 NG-tubes, 89% yielded more than 1,000 colony-forming units (CFU)/ml bacteria, and 55% yielded the potentially pathogenic *Enterobacteriaceae* and/or *Staphylococcus aureus*. The mean concentration in the yield was 5.3 (SD: 2.1, maximum 9.4) log₁₀ CFU/ml. Neither the presence of contamination nor the density was associated with the time the NG-tube had been in use. Probiotic administration did not protect against contamination.

CONCLUSION: NG-tubes yielded high densities of bacteria even within the first day of use. Further studies are needed to determine if changing the NG-tubes between meals or once a day will make a positive impact on tube contamination and clinical parameters.

The gut of a preterm infant is immature regarding immunological defense, digestive functions, and motility (1–3). When admitted to a neonatal department, the infant is colonized with bacteria of the environment (4), and the dysbiosis that might occur combined with the immaturity of the gut can lead to feeding intolerance, sepsis, or necrotizing enterocolitis (NEC) (5).

Nasogastric feeding tubes (NG-tubes) probably play a role in the exchange of bacteria between the environment and the infant (4). Preterm infants are dependent on NG-tubes for weeks or even months. The NG-tube is exposed to bacteria from unpasteurized milk, from the gut of the infant, or from the hands of parents and personnel, and a biofilm with potential pathogens has been shown to develop in NG-tubes of newborn infants (6,7). A recent quality improvement study which

showed a reduction in NEC incidence, reported that one of their interventions was to renew NG-tubes once a week (8). Greater bacterial counts were found in the static biofilm of NG-tubes that had been in use for more than 48 h (6), but it was not investigated how many viable bacteria from the biofilm, if any, were actually flushed into the stomach and upper gastrointestinal tract of the infant when a meal was given through the tube. In another observational study, however, formula-fed infants with contaminated NG-tubes suffered from more feeding intolerance and necrotizing enterocolitis (NEC) than infants with clean NG-tubes (7). In that study, infants who received breast milk did not suffer from any feeding intolerance or NEC, in NICUs using human milk, nevertheless, feeding intolerance and NEC may still be a considerable problem (9).

Considering that renewing a NG-tube causes measurable distress in preterm infants (10), we wanted to determine if NG-tubes yielded less bacteria to the infant, the shorter they had been in use. Furthermore, since the use of probiotics for preterm infants to prevent NEC and sepsis is gaining ground (11), and since *Lactobacillus* spp. were shown to disrupt biofilms (12), we also wanted to determine if administration of probiotics through the tube had any limiting effect on the bacterial output from the tubes. Thus, we conducted an observational study of used NG-tubes from infants of the neonatal department to study the labile part of the biofilm. We flushed a saline solution through the NG-tubes, investigated the presence and the density of bacteria in this imitated meal, and related it to the duration of time the NG-tube had been in place in the infant as well as to probiotic administration through the tube when in place.

RESULTS

Bacterial Isolates From the Flush of NG-Tubes

In the period from April to June 2014, we cultured the flush of 94 nasogastric feeding tubes from 34 infants (range: 1–9 NG-tubes per infant (median = 2)). The infants had a median gestational age of 30.1 wk and a birth weight of 1,083 g (Table 1). The tubes had been in place for a median of 3.25 d (range = 8 h to 14.2 d), and the postnatal median age at the time of collection was 37 d (range = 1–119 d). The reason for renewing the NG-tube was mainly that it was accidentally pulled

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Received 21 October 2015; accepted 16 February 2016; advance online publication 11 May 2016. doi:10.1038/pr.2016.86

Table 1. Clinical data of the infants (n = 34) and nasogastric feeding tubes (n = 94)

At birth	
Birth weight (g), median (IQR)	1,083 (1,259)
Gestational age (weeks), median (IQR)	30.1 (7.6)
Male sex	20 (58.8)
Cesarian section	22 (64.7)
Multiple birth	14 (41.2)
While the tube was in place	
Postnatal age (days), median (IQR)	37 (39)
Unpasteurized maternal milk feeding	85 (90.4)
Probiotics ^a	72 (76.6)
Antibiotics	42 (44.7)
Proton pump inhibitor	1 (1.1)
Ventilation	
Mechanical	8 (8.5)
Nasal c-pap	57 (60.6)
Oxygen mask	3 (3.2)
None	26 (27.7)
Parenteral nutrition	
Major outcomes	21 (22.3)
Sepsis ^b	5 (14.7)
Necrotizing enterocolitis ^c	6 (17.6)
Deceased	4 (11.8)

Data are presented as n (%) unless otherwise indicated. ^a*Lactobacillus rhamnosus* GG and *Bifidobacterium animalis* ssp. *Lactis*. ^bPositive blood culture and clinical signs of sepsis. ^cBells modified stage II or III.

out (53%), routine practice (27%), and clotting of tube (20%). We identified 178 isolates with a colony-forming units (CFU) count greater than 1,000 CFU/ml, a median of two isolates per tube (range 0–5).

Four NG-tubes yielded culture-negative flushes, four yielded only isolates of less than 1,000 CFU/ml, and two yielded only *Lactobacillus rhamnosus* or *Bifidobacterium animalis*. Hence, the flush of the remaining 84 NG-tubes (89%) were considered contaminated. The most common bacteria were coagulase-negative *Staphylococci* and *Enterococcus spp.* (Table 2). Potentially pathogenic bacteria (Gram-negative rods and *Staphylococcus aureus*) were isolated from the flush of 52 NG-tubes (55%) originating from 22 infants (65%). *Klebsiella* and *Enterobacter spp.* were cultured most frequently. The NG-tubes yielded a mean of 5.3 (SD 2.1) log₁₀ CFU/ml bacteria with a maximum count of 9.4 log₁₀ CFU/ml.

Duration of Use and Bacterial Contamination

The presence of bacteria was not associated with the number of days the NG-tube had been in use. NG-tubes that yielded a contaminated flush, had been in use for a median of 3.4 d interquartile range (IQR = 4.9) compared to 2.2 d (IQR = 3.2) for NG-tubes that did not (P = 0.18 Mann-Whitney U-test). NG-tubes that yielded a flush with potentially pathogenic

Table 2. Bacterial isolates and their concentration in the yield of used nasogastric feeding tubes collected in a tertiary neonatal department

Isolate	NG-tubes, n (%)	Infants, n (%)	Concentration, mean ± SD
	(n = 94)	(n = 34)	Log10CFU/ml
CoNS	44 (46.8)	23 (67.6)	4.6 ± 1.3
<i>S. epidermidis</i>	33 (35.1)	21 (61.8)	4.6 ± 1.3
<i>S. haemolyticus</i>	14 (14.9)	10 (29.4)	4.9 ± 1.5
<i>S. hominis</i>	5 (5.3)	4 (11.8)	4.5 ± 0.95
Unspecified	7 (7.4)	6 (17.6)	4.1 ± 0.86
<i>Enterococcus sp.</i>	25 (26.6)	13 (38.2)	5.5 ± 1.4
<i>E. faecium</i>	15 (16.0)	5 (14.7)	6.0 ± 1.5
<i>E. faecalis</i>	10 (10.6)	8 (23.5)	4.8 ± 1.2
<i>Klebsiella sp.</i>	20 (21.3)	7 (20.6)	5.9 ± 1.8
<i>K. oxytoca</i>	15 (16.0)	5 (14.7)	6.0 ± 1.8
<i>K. pneumoniae</i>	7 (7.4)	4 (11.8)	5.5 ± 1.9
<i>Enterobacter cloacae complex</i>	19 (20.2)	5 (14.7)	6.0 ± 1.7
<i>Lactobacillus rhamnosus</i>	14 (14.9)	10 (29.4)	6.4 ± 1.8
<i>Staphylococcus aureus</i>	13 (13.8)	10 (29.4)	4.8 ± 1.0
Alpha-hemolytic <i>Streptococcus</i>	10 (10.6)	7 (20.6)	4.3 ± 1.2
<i>Micrococcus lutea</i>	5 (5.3)	5 (14.7)	4.0 ± 0.9
<i>Escherichia coli</i>	3 (3.2)	3 (8.8)	6.0 ± 2.9
<i>Serratia marcescens</i>	2 (2.1)	2 (5.9)	4.7 ± 1.9
Yeast	2 (2.1)	2 (5.9)	3.4 ± 0.12
<i>Bifidobacterium animalis</i>	1 (1.1)	1 (2.9)	3.6
<i>Neisseria mucosa</i>	1 (1.1)	1 (2.9)	4.3
<i>Rothia mucigalinos</i>	1 (1.1)	1 (2.9)	5.2
<i>Bacillus cereus</i>	1 (1.1)	1 (2.9)	3.0

CoNS, coagulase-negative *Staphylococci*.

bacteria had been in use for a median of 3.7 d (IQR = 4.9) compared to a median of 3.2 d (IQR = 5.1) in tubes that did not (P = 0.54 Mann-Whitney U-test).

There was no correlation between the number of days the NG-tube had been in use and the quantity of contamination with all bacteria (CFU/ml) (Spearman correlation r_s = 0.065, P = 0.53), or the quantity of contamination with potentially pathogenic bacteria (Spearman correlation r_s = -0.11, P = 0.43) (Figure 1). There was, however, a weak positive correlation between infant age at NG-tube collection and the quantity of contamination with potentially pathogenic bacteria (all bacteria; r_s = 0.15, P = 0.16, and potential pathogens; r_s = 0.29, P = 0.036).

Probiotic Use

Probiotics (*Lactobacillus rhamnosus* GG and *Bifidobacterium animalis* ssp. *lactis*) were given to 20 (59%) of the infants, and 73 (78%) of the NG-tubes had been used for probiotic administration. We isolated probiotic bacteria from 15 (21%) of these and from none that had not been used for probiotic administration (P = 0.02 Fishers exact test). The administration of

probiotics through the NG-tubes was not associated with the risk of contamination (88 vs. 90% of probiotic and nonprobiotic NG-tubes, respectively), nor contamination with potential pathogens (56 vs. 52% of probiotic and non-probiotic NG-tubes, respectively).

Antibiotic Use and Susceptibility

We observed that 42 (44.7%) of the collected NG-tubes were taken from infants who received intravenous antibiotics while

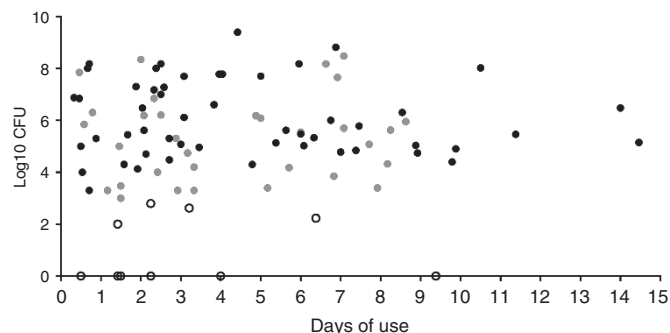


Figure 1. Number of days the nasogastric tube was in use plotted against the concentration of bacteria in a 1-ml yield from the used nasogastric feeding tube (except probiotic strains). White circles: Not identified/no bacteria. Gray circles: No potential pathogens. Black circles: Potential pathogens.

the tube was in place. There was no statistical significant association between intravenous antibiotic use and colonization of the NG-tubes with all bacteria ($P = 0.33$ Fisher's exact test) or with potential pathogens ($P = 0.077$ Chi-squared). There were no multidrug-resistant bacteria among the *Enterobacteriaceae* in our samples (data not shown).

Visualization of Biofilm by Scanning Electron Microscopy

We visualized the inner surface of the NG-tube with scanning electron microscope that showed that a dense biofilm was present in both the proximal and the distal (intra-gastric) part of the NG-tube (Figure 2). We found a mixed bacterial community with cocci, rods, and yeast, but the specimens also revealed the presence of macrophages on the inner surface (Figure 2b).

DISCUSSION

We found that great majority of NG-tubes yielded high counts of bacteria when flushed with a test meal. The most common bacteria were *Enterococcus* spp. and coagulase-negative *Staphylococci*, but half of the tubes yielded potentially pathogenic bacteria. Neither the presence of contamination nor the density was associated with the number of days the NG-tube had been in use and probiotic administration did not seem to protect against contamination. Hence, the majority of the infants were exposed to a considerable dose of live bacteria

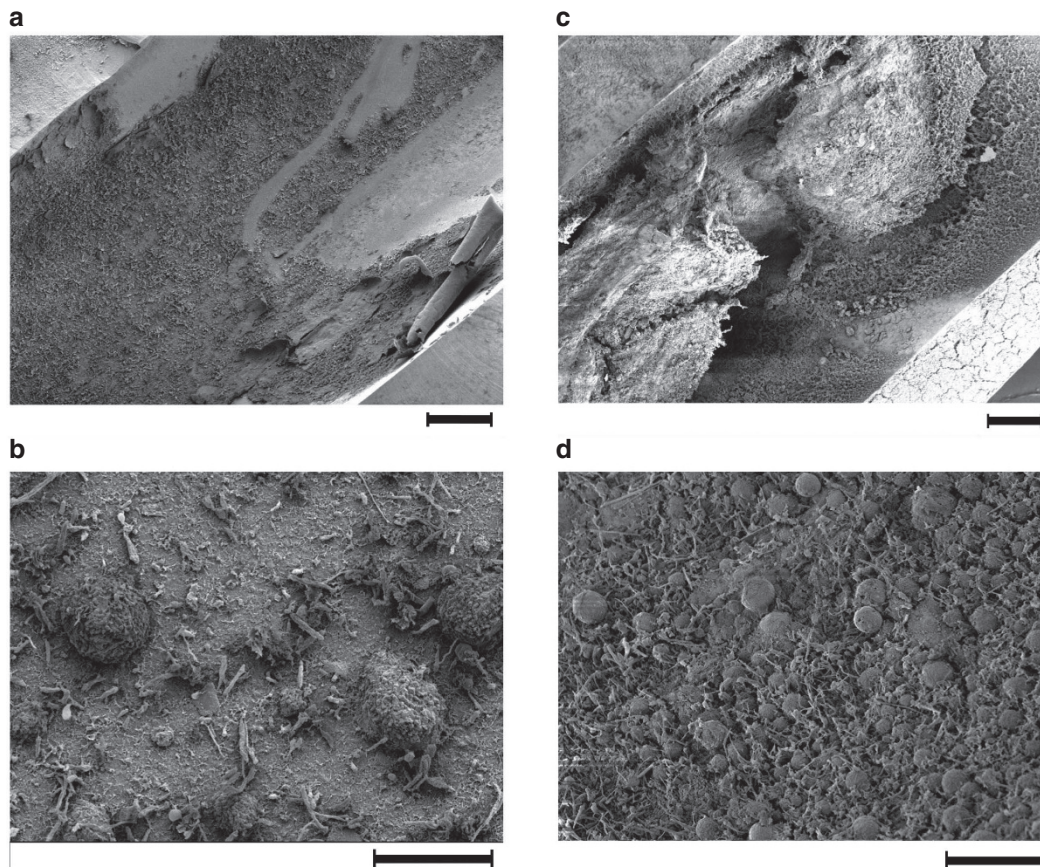


Figure 2. Scanning electron microscopy images of the interior surface of a used nasogastric feeding tube from a preterm infant admitted at the neonatal department. The proximal part (a, b) and the distal (intra-gastric) part (c, d) of the feeding tube is visualized. Magnifications: (a) 80 \times , (b) 1,500 \times , (c) 60 \times , (d) 1,500 \times . Scale bars: (a) 200 μ m, (b) 20 μ m, (c) 200 μ m, (d) 50 μ m.

every time they were fed through a resident NG-tube, even within the first day of use.

The most frequently isolated bacterial species were similar to those previously described in the biofilm of NG-tubes (7). Hurrell et al. (6) found that NG-tubes that had been in use for less than 6 h contained significantly lower bacterial counts of *Enterobacteriaceae* in the biofilm than those in use for a longer period of time and reported increasing counts in the first 48 h. However, by vortexing and ultra-sonicating tube pieces after removing residual liquid from the tube, they did not investigate the yield of the NG-tubes, only the biofilm attached to the NG-tube. It is expected that the biofilm inside a NG-tube grows thicker over the first 24–48 h of use (13), but the yield of bacteria may not change over time.

We found a weak correlation between bacterial contamination of the NG-tubes and infant age at collection. Similar bacterial species were isolated from NG tubes of the same infants. Furthermore, *Enterobacteriaceae* isolated from several tubes of the same infant exhibited the same patterns of antibiotic resistance. Hence, our findings indicate that the bacteria originated from the infant. We speculate that the bacteria of the stomach enter the feeding tube when gastric content is aspirated to check for residuals before each meal.

Only few groups have investigated the early colonization of the upper part of the gastrointestinal tract in preterms, but they found that the microbiomes of gastric aspirates are unstable and consisting of low bacterial counts (14–16). The total number of bacteria decreased over the first 4 wk in one study where *Bacteroides* and *Escherichia coli* were predominant (14). On the contrary, in another study, the total number of bacteria increased and *Staphylococcus epidermidis* was the main colonizer (15). Duodenal aspirates from preterm infants contained none or few Gram-positive bacteria in the first days of life with increasing probability of colonization with *Staphylococci*, *Enterococci*, and Gram-negative bacteria, especially *Escherichia coli*, and *Klebsiella pneumoniae* and *K. oxytoca*, over the first weeks. The density of Gram-negative bacteria were approximately 4.0 log₁₀CFU/g (16). This means that giving 10⁸ CFU of *Enterobacteriaceae* with a 1-ml meal is an enormous amount compared to what might already be present in the upper gastrointestinal tract of the infant.

Routine evaluation of gastric residuals is common practice but not evidence based, and a randomized-controlled trial to evaluate if routine gastric aspiration is a beneficial or perhaps a detrimental practice is underway (17). One mechanism of harm could be by leading to multiplication of bacteria in the NG-tube. Other potential sources of the colonization of the tube are the nasal or pharyngeal microbiome (18), unpasteurized maternal milk (19), or the skin of parents and/or personnel.

We isolated the probiotic strains (*Lactobacillus rhamnosus* or *Bifidobacterium animalis*) in 20% of the NG-tubes from infants who received probiotic supplementation. We did not find probiotic strains in NG-tubes of infants who did not receive probiotics in the period where the NG-tube had been in place, even though some cross contamination with probiotics is known to

occur (20). Due to the study design, it remains uncertain if the probiotic strains were a part of the biofilm or were present in the residual liquid in the NG-tube. Theoretically, they could derive from a recent administration of probiotics through the tube. Probiotic supplementation for preterm infants is still a controversial topic (21), and this study did not provide any evidence that probiotics could be beneficial due to an inhibitory effect on biofilm formation in NG-tubes.

We did not find any multidrug-resistant *Enterobacteriaceae*, which reflects the situation in Denmark where multidrug resistance is relatively uncommon (22). However, this might be a problem in other parts of the world where multidrug-resistant bacteria are more common. If bacteria that carry resistance genes are able to amplify to a high concentration in a biofilm in the NG-tube, they may transmit resistance genes to other bacteria when they are entering the gut (23).

As expected, a dense biofilm was visualized on the inner surface of the NG-tubes with scanning electron microscopy. The biofilm looked thicker in the lower part of the NG-tube, which contributes to the theory that the biofilm mostly consists of bacteria from the gut of the infant. Unexpectedly, we saw macrophages on the inner surface of the NG-tube. We speculate that they may derive from the maternal unpasteurized milk given through the tube, but their role here is not known.

Strengths and Weaknesses

This is to our knowledge the first study to investigate bacterial counts in imitated meals given through used NG-tubes. In a neonatal department where the patients are vulnerable, high counts of bacteria may be clinically important. We used culture techniques in order to investigate the viable part of the microbiome inside the NG-tube and to determine the CFU/ml of each isolate and the antibiotic resistance patterns of the Gram-negative rods. This, of course, comes at the price of missing bacteria that are detectable by molecular methods only (24). As an example, we isolated the probiotic bacterium *Bifidobacterium animalis* in only one of our samples, but *Lactobacillus rhamnosus* in fourteen, even though they were given as a mixture to the infants. If this is due to the difficulty of culturing the strict anaerobic *Bifidobacterium* or a true difference in the presence of the two probiotic bacteria in our samples, remains unknown. Furthermore, the study was purely observational, and the NG-tubes were collected as a convenience-sample.

There was a high proportion of infants with NEC in our population (17.6%), much higher than our last reported rate of 6% (9). This is simply because infants with NEC are hospitalized for longer periods of time and hence were more likely to be included in our convenience-sample study. Because of the small number of infants, we did not formally statistically examine the relation of NG-tube contamination to NEC or death. Furthermore, from simple observation of the data, there was no apparent pattern.

We stored the NG-tubes in a refrigerator for 1–3 (up to 5) days after removal from the infant, but NG-tubes has previously been stored at 4 °C for up to 8 d without affecting the

results of the cultures (7). Since we investigated a saline solution flushed through the tube, our sample also contained the residual liquid inside the tube. The bacterial count in this liquid might have differed depending on the time span between last feeding and removal of NG-tube, especially if the last meal was unpasteurized maternal milk which 90% of the infants received. However, the standard at the department was to remove the NG-tube just before giving the next meal, thus the test meal reflects normal practice. We did not examine the bacterial counts in subsequent flushes, or larger volumes of flush, but the bacterial counts we report must be a minimum, compared to those typically given as part of normal enteral feeding in preterm infants.

Perspectives

The high bacterial counts delivered from the NG-tubes give rise to two main concerns. The first concern is if NG-tubes are handled with sufficient hygienic precautions, considering their potential as a reservoir for pathogenic bacteria. Nosocomial colonization of preterm infants with *Klebsiella oxytoca* in a neonatal department was associated to the handling of the NG-tubes and was eliminated by reinforcement of glove-usage when inserting NG-tubes and feeding infants through these (25). Our findings and the existing literature suggest that resident NG-tubes should be handled with the same hygiene standards as materials containing fecal matter.

Second concern is if contamination of feeds given through resident NG-tubes affects the health of the preterm infants and other vulnerable patients. Development of antimicrobial NG-tubes with silver-impregnated inner surfaces has not been successful in avoiding biofilm formation (13), and our findings show that replacing the NG-tube more often, e.g., once a day, will not eliminate bacterial contamination of feeds. So, the question remains if the benefits of a resident NG-feeding tube outweigh the potential harms. An alternative would be to insert a new or decontaminated NG-tube for every meal. Considering the potential risks of inserting a NG-tube (26), we are currently planning a randomized controlled trial to investigate if changing the NG-tubes once a day in the first week of life, can reduce the concentration of bacteria in the stomach of the infant on day 7. We also plan to further study the spontaneous colonization of the upper gastrointestinal tract in this vulnerable population. If frequent replacement of NG-tubes can reduce bacterial concentration in the stomach, a large, multicenter study with clinical end-points as feeding intolerance or NEC should be planned.

Conclusion

Used NG-tubes from infants in a neonatal department often yielded high densities of bacteria, and administration of probiotics through the tube did not limit this. Neither the presence of bacteria nor the density was associated with the duration of time the NG-tube had been in use, and bacterial counts were high even within the first day. Further studies are needed to determine if changing the NG-tubes between each meal or once a day will make a positive impact on NG-tube

contamination, clinical parameters, such as feeding intolerance, growth, or even NEC-incidence.

METHODS

Study Population

We conducted a prospective, observational study at the tertiary neonatal department, Rigshospitalet, Copenhagen, Denmark. Inclusion criteria were admission to the neonatal department and a resident NG-tube at the time of inclusion. The study was approved by the Danish Ethical Committee (Protocol number: H-1-2014-009), and written informed consent was sought from one of the parents according to Danish law. In the 2-mo study period from April to June 2014, we collected and cultured the used NG-tubes of included infants, and at the end of the study period, we collected clinical data from the infant files. This was baseline data of the infant (delivery mode, gestational age, sex, birth weight, multiple birth), data for each NG-tube placement-period (infant postnatal age at collection of tube, nutrition, type of ventilation, use of probiotics), and finally data on diagnoses during the hospitalization (occurrence of necrotizing enterocolitis, sepsis and/or death).

Feeding Practices at the Department

The policy of the department is to change the NG-tube weekly and more often when necessary (if clotted or misplaced). A meal is given by attaching a 10-ml syringe to the NG-tube. Before a meal is given, gastric content is aspirated to check for residuals from last meal, and then milk is poured into the open syringe and passed passively through the tube by gravity. When the milk has passed, a small amount of air is pressed through the NG-tube to clear it for residual liquid. Meals are given every 2–3 h by the nurse or the parents of the infant. Parents and personnel are encouraged to use disposable gloves when feeding, and parents receive training before administering NG-feeds to their infant. The infants are given raw mother's milk as the first choice and if insufficient in amount, it is combined with pasteurized donor milk or preterm formula depending on gestational and postnatal age. Infants with a gestational age < 30 wk are given probiotics (two capsules of 10^9 CFU *Lactobacillus rhamnosus* GG (LGG) + 10^8 CFU *Bifidobacterium animalis* ssp. *lactis* (BB12)) from day 3 of life. Probiotics are given through the NG-tube after dissolution into the feeds. In the study period, we used Kangaroo polyurethane feeding tubes (Covidien, Dublin, Ireland) of different sizes depending on the size of the infant.

Feeding Tube Collection

When the NG-tube of an included infant was renewed, the used NG-tube was placed in a labeled plastic bag and stored in a refrigerator at 4 °C. The plastic bag was labeled with patient ID, date of collection, date of placement in infant, and reason for changing tube ("routine", "clotted", "accidentally displaced", or "other"). Duration of NG-tube use was recorded according to the date of insertion and collection. Exact time of the insertion and collection was retrieved from the records of the infant, and if no record was found of the exact time, it was assumed that the tube was inserted/collected at noon. The NG-tube was kept in the refrigerator and transferred to the laboratory for culturing within 1–3 d (few were stored up to 5 d).

Bacteriological Analyses

In the laboratory, the NG-tubes were flushed with 1 ml NaCl to mimic a 1 ml meal given through the NG-tube. The flush was cultured directly and by serial ten-fold dilutions for 24 h at aerobic conditions on blood agar plates and SSI enteric plates, for 48 h at 5% CO₂ on chocolate plates and for 48 h at anaerobic conditions on anaerobe plates (all growth media were obtained from SSI Diagnostika, Hillerød, Denmark). The unique colonies were counted and isolated for further identification. Isolates were stored at –80 °C.

We defined a count of less than 1,000 CFU/ml in the flush as clinically unimportant. Hence, all isolates with a count of more than 1,000 CFU/ml were identified by the VITEK 2 system (Biomérieux, Marcy l'étoile, France) complemented with standard laboratory methods or MALDI-TOF (MALDI Biotyper, Bruker, Billerica, MA), when

the VITEK system failed to provide an acceptable identification. We tested Gram-negative rods for antibiotic susceptibility by the disc diffusion method according to European Clinical Antimicrobial Susceptibility Testing guidelines (27). We tested with the following Neo-sensitabs (Rosco Diagnostika, Taastrup, Denmark): Ampicillin (33 µg), Cefuroxime (60 µg), Cefotaxime (30 µg), Gentamicin (10 µg), Piperacillin-Tazobactam (30 µg + 6 µg), Sulfamethoxazole-Trimethoprim (240 µg + 5.2 µg), Ciprofloxacin (5 µg), and Meropenem (10 µg).

Visualization of Biofilm by Scanning Electron Microscopy

To visualize the presence of a biofilm on the interior surface, we flushed a used NG-tube with 1 ml saline to remove residual liquid and cut 1-cm pieces from the proximal part and the distal (intragastric) part of the tube which we then dissected longitudinally to reveal the inner surface. The specimens were fixed in 2% glutaraldehyde in 0.05 M sodium phosphate buffer of pH 7.4. Following three rinses in 0.15 M sodium phosphate buffer (pH 7.4), specimens were postfixed in 1% OsO₄ in 0.12 M sodium cacodylate buffer (pH 7.4) for 2 h. Following a rinse in distilled water, the specimens were dehydrated to 100% ethanol according to standard procedures and critical point dried (CPD 030, Leica, Wetzlar, Germany) employing CO₂, and the specimens were subsequently mounted on stubs using colloidal coal as an adhesive, and sputter coated with gold (Leica Coater ACE 200). The inner surface of the NG-tube was then examined with a FEG30 scanning electron microscope (Phillips medical systems, Eindhoven, The Netherlands) operated at an accelerating voltage of 2 kV to visualize the presence of a biofilm.

Statistics

A NG-tube was defined as contaminated if it yielded more than 1,000 CFU of at least one isolate, except for the probiotics supplemented to the infants. Potentially pathogenic bacteria were defined as Gram-negative rods and *Staphylococcus aureus*. The distribution of variables was not normal, thus the Mann-Whitney *U*-test test was used to assess association between categorical and continuous variables, Chi-squared or Fisher's exact test was used for categorical variables, and the Spearman Rank order correlation for continuous variables. Statistical significance was set at $P < 0.05$. Calculations were made in SPSS Statistics v. 20 (IBM, Armonk, NY).

ACKNOWLEDGMENTS

Thanks to the nurses at the neonatal department for collecting the nasogastric tubes, and to Susanne Schjørring, Michala Sørensen, Rie Jønsson, Christina Tingbjerg Brandt, Steffen Jørgensen, and Maj Holm for their contributions in the laboratory at Statens Serum Institute. We acknowledge the Core Facility for Integrated Microscopy, Faculty of Health and Medical Sciences, University of Copenhagen.

STATEMENT OF FINANCIAL SUPPORT

This study was financially supported by the NEOMUNE grant from the Danish Research Councils and by the Dagmar Marshall Foundation, Copenhagen, Denmark.

Disclosures: All authors declare no conflict of interest.

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