Title: Optimal Feeding Tube Dwell Time in VLBW Infants to Reduce Feeding Tube Contamination

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Protocol

4. Background:

The long-term goal of this research is to improve short and long-term health outcomes for premature infants in the NICU by decreasing complications caused by contaminated feeding tubes such as sepsis, pneumonia and feeding intolerance. Because premature infants in the NICU may be too immature or ill to orally feed, a feeding tube is required to meet their nutritional needs. These feeding tubes have been shown to be a reservoir for pathogenic and often antibiotic-resistant bacteria and are thus a risk to neonatal health.

Contaminated feeding tubes are a conduit for bacterial migration into the stomach and intestines leading to gastrointestinal pathogenic bacterial overgrowth, abnormalities in pro-inflammatory cytokine response and increasing the risk of late onset sepsis, necrotizing enterocolitis and feeding intolerance. Feeding tubes also disrupt the oral microbiota increasing pathogenic mouth bacterial and the risk of pneumonia. The critically ill infant's significantly suppressed immunity, impaired intestinal barrier function and lack of beneficial gastrointestinal bacteria place them at tremendous risk of morbidity related to feeding tube contamination.

Contamination of medical devices such as feeding tubes is related to length of time the device remains in place (dwell time). In the NICU, universal guidelines regarding feeding tube dwell time are non-existent and feeding tube replacement is often delayed for weeks following insertion. The optimal feeding tube dwell time to reduce or prevent contamination and its associated morbidity is currently unknown. In addition, the optimal feeding tube dwell time to avoid abnormal bacterial colonization of the stomach and intestines and the extent to which contamination of these domains relate to adverse neonatal health outcomes is unknown. Thus, the primary purpose of this study is to determine if a maximum feeding tube dwell time of 48 hours reduces contamination compared to a feeding tube dwell time of 7 days, thereby improving neonatal health outcomes.

Aims include the following:

Primary Aim: To determine if a 48 hour maximum dwell time reduces bacterial contamination and biofilm formation of feeding tubes, feeding tube hubs, and intraluminal fluid in premature VLBW infants. **Primary hypothesis**: Feeding tubes which remain in place 0-48 hours will have less contamination and less biofilm formation compared to those left in place for 7 days.

Aim 2: Compare gastrointestinal microbial dysbiosis and inflammation between Group 1 and Group 2. **Hypothesis:** Infants in Group 1 will have less GI microbial dysbiosis and inflammation compared to Group 2.

Aim 3: Determine the effect of feeding tube dwell time and feeding tube contamination, and biofilm formation on selected health outcomes in premature VLBW infants. **Hypothesis:** Infants in Group 1 will have lower odds for necrotizing enterocolitis and late onset sepsis, and improved nutritional outcomes compared to Group 2.

5. Research Plan:

A randomized controlled trial will be conducted to determine whether feeding tube dwell time is associated with level of feeding tube contamination. Infants will be randomly assigned to have their feeding tubes removed at 0-48 hours or 7 days for the first 4 weeks of life. The feeding tube lumen, interluminal liquid and hub will be analyzed for level of contamination.

Sample and Setting

The proposed study will follow a prospective cohort (N =151) premature VLBW male and female infants for 4 weeks following birth. If the mother is providing breast milk to her infant, she will also be included in the study since she will be providing 2 samples of her breast milk for analysis.

Inclusion criteria are: 1) be born to a mother who is at least 18 years of age and English or Spanish speaking, 2) born at ≤ 30 weeks 3) have a birthweight ≤ 1500 grams, 4) be born to a mother who is COVID Negative, 5) have a feeding tube placed within 24 hours of birth and 5) be expected to require a feeding tube for 4 weeks following birth.

Exclusion criteria are: 1) Infant with known congenital GI anomalies, (2) are not expected to live, or (3) Infants will be withdrawn from the study if they require abdominal surgery for GI morbidities.

Sample Size Determination/Power Analysis: The primary aim of this study is to test for associations between feeding tube dwell time and the bacterial load present in the feeding tube. Pilot study results were used to estimate variance for the log transformed values of bacterial load observed in cultures. The study employs a repeated measures design, with measurements performed on each neonate each week of the 4 week study duration. For the primary bacterial contamination aim, each measurement consists of measures from 5 individual feeding tube sections for 5 individual colony types, providing a total of 100 within-subjects data points per neonate. Given the repeated measures design and the incorporation of selected covariates, a Generalized Linear Mixed Model (GLMM) analysis is planned for the primary aim. The data derived from the pilot study of 31 feeding tubes informed the covariance structure and means vectors for entry into PASS 14's procedure which estimates mixed models power using simulation. Using a .005 level of significance (in recognition of the multiple inferential tests), 400 simulation samples, a minimum detectable difference of 3.0 (natural log transformed colony forming units), and employing the pilot data to inform mean vectors and the unstructured covariance matrix for the simulations, the lower 99% confidence limit for power is .97 for the Dwell Group by Colony Type interaction, which is of primary interest. To accommodate the anticipated 10% loss due to surgery or death, 134 neonates will be enrolled. If mothers are providing breast milk samples, 151 mothers would also be enrolled.

Standard Infant Feeding Protocol

As per standard NICU protocol, all infants will begin receiving TPN within 24 hours of life. In addition, all infants will have an orogastric (OG) or nasogastric (NG) tube placed upon admission to the NICU and begin receiving some enteral feeding within 72 hours. Length of insertion of the OG/NG tube is determined by measuring from the tip of the nose to the tip of the ear lobe and then halfway between the xyphoid process and umbilicus (Ellett et al., 2011). Minimum insertion length based on the weight of the infant (Cordero et al., 2011). If the infant receives an X-ray upon admission, placement of the OG/NG tube is verified. Placement is also verified if additional X-rays are taken during the study as per usual OG/NG placement protocol in the NICU. Depending on the infant's gestational age, weight, and clinical status, feedings are initiated at up to 20mL/kg/d and advanced daily by no more than 20mL/kg/d toward a goal of 120-150mL/kg/d divided into 8 equal feedings per day using the established University of Florida Children's Hospital NICU feeding guidelines. If mothers own breast milk is unavailable, infants will be provided donor breast milk as per NICU standard care.

Consent Process

Within 72 hours of life, and within 24 hour of initiating feeds of ≤ 20 mL/kg/d, informed consent will be obtained from the mother either in her hospital room or the NICU. The PI or research coordinator will ask the mother's or infant's nurse whether the mother is willing to speak with someone regarding enrolling her infant in a research study. If the mother says yes, the PI or research coordinator will explain the study and invite the mother to have her infant enrolled. All questions will be answered and mothers will be provided ample time to discuss with family and friends. If the mother consents and is providing breast milk to her infant, she will also be consented since will be providing 2 samples of breast milk for analysis

Breast Milk Feedings

Current practice in this NICU is to provide donor breast milk to all infants born prior to 30 weeks until they are 32 weeks post conceptual age. For this study, all infants will receive either mother's own breast milk or donor human milk for the 4 week study. If the infant is scheduled to be discharged prior to 4 weeks, the infant will be transitioned to formula as per NICU standards.

Procedure

Following consent, data including gestational age, chronologic age, birth weight, race, gender, date and time of sample collection, feeding tube dwell time, duration of antibiotic therapy since birth, episodes of sepsis, other medications, mode of delivery, and mode and type of feeding will be obtained from the EMR. Other data including incidence of stage 2 or greater necrotizing enterocolitis; episodes of presumed or culture proven late onset sepsis (occurring > 3 days of life); days to discharge; nutritional outcomes, including weekly enteral intake, days to full enteral feedings (120 mL/kg/d),(150ml/kg/day), hours of parenteral nutrition, evidence of PNALD (level of direct bilirubin, alkaline phosphatase, aspartate aminotransferase (AST) and alanine aminotransferase (ALT); growth indices (weekly weight, head circumference and length); and hours with a central venous line will be collected.

Infants in both groups will have a bedside sign and EPIC note stating which group they are assigned to and when they are scheduled for feeding tube replacement. Infants will have their feeding tubes replaced 0-48h (Group 1) and every 7 days (Group 2). At the designated time, the feeding tube will be replaced by either the bedside nurse or the research coordinator (an experienced NICU nurse) using the NICU's standard method of feeding tube insertion.

All tubes will be adhered to the infant's face using the same procedure designed to decrease the risk of feeding tube dislodgement and protect skin integrity. The method of tube securement will be approved by NICU management/staff and comply with NICU skin care guidelines. The orogastric tubes will be taped to the chin from the middle of the mouth and nasogastric tubes will be taped under the lateral edge of nares. The research team will check the security of the tube adherence 3x per week and re-secure as necessary. On the day that the study feeding tube is scheduled to be removed, the research coordinator will coordinate with the bedside nurse when the feeding tube will be removed and replaced. In the event the infant has a Replogle tube instead of a feeding tube on lab collection day, the Replogle tube will collected. At the designated time, the feeding tube or Replogle will be removed and immediately placed in a sterile Ziploc bag and placed on ice for immediate transfer to laboratory by member to research team.

Microbial Culturing and Analysis After feeding tubes are removed, the tube will be divided in three portions; 1) the inner (stomach) intra-corporeal portion, 2) the inner intra-corporeal portion, and c) the extracorporeal portion. The external surface of the tube will be cleaned with isopropyl alcohol. The outside of the tube will be rolled across 5% sheep blood agar and McConkey's at two separate points as a control for external contamination. Using sterile technique, the tube

then will be sectioned into 5-cm portions and submerged in 20 mL sterile saline in a conical test tube (we will probably add in another cutting step in order to split the tubes open using a sterile scalpel). This will be vortexed on high for 1 minute. The saline will be decanted into a second sterile test tube, leaving the tube pieces in the first test tube. Another 20 mL aliquot of sterile saline will be placed in the first test tube, and this was vortexed and will be decanted into the second test tube. The decanted saline will then be centrifuged at 8,000 rpm for 10 minutes. After centrifugation, the supernatant will be discarded and the pellet resuspended in 1 mL sterile saline. Serial 10-fold dilutions will be performed, each will be plated onto both 5% sheep blood agar and McConkey's agar. Incubated up to 48 hours at 37 C. After 24 hours of incubation, unique colony types will be counted. We will also isolate colonies by replating onto their respective medium, and reincubated. (Original plates were reincubated an additional 24 hours and reassessed for unique colony types). After isolation, each organism was identified and frozen at -70 C in glycerol trypticase soy broth.

DNA Extraction and 16S rRNA sequencing. DNA will be extracted from cells pellets obtained after tube processing. DNA will be extracted using PowerFecal DNA isolation kit (MO BIO Laboratories). DNA will be quantified using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE). V4 universal primers will be used for 16S rRNA amplification and subsequent Illumina MiSeq sequencing. Samples will be amplified in 25 µl reactions containing 0.5 Units Phusion High-Fidelity Polymerase (New England Biolabs, Ipswich, MA), 1X Phusion HF Reaction Buffer, 0.75 µl DMSO, and 0.2 mM each dNTP. Triplicate PCR amplifications will be pooled for each sample, and cleaned with a QIAquick PCR Purification kit (Qiagen). Two hundred nanograms of each cleaned amplicon library will be submitted for sequencing at the ICBR core facility at the University of Florida. Sequencing will be performed on an Illumina MiSeq with a 300-bp paired-end protocol, using single indexing.

Microbiome 16S rRNA Gene Analysis. Sequencing reads will be parsed by Illumina index at the sequencing center. Paired reads will be merged, and primers and adaptors will be removed using a combination of tools in cutadapt, Galaxy, and eautils. Parsed raw sequencing reads will be made available to the scientific community after publication through NCBI's Sequence Read Archive (SRA). Sample names will be added to the definition lines of sequencing reads using sed and concatenated into one fasta file, to make them compatible for analysis in QIIME v1.9. Clustering of Operational Taxonomic Units (OTUs) at 97% similarity will be performed with the subsampled open-reference OTU picking method, with no removal of singletons. The Greengenes reference dataset version 13.8 will be used as the reference for OTU picking and for taxonomy assignment with uclust. Community structure will be analyzed in R with phyloseq and will be plotted with ggplot2. Analysis of similarities (ANOSIM) will be performed in R using VEGAN v2.0-8. Statistical significance of QIIME profiles will be assessed using STAMP.

Metagenomic analyses. For metagenomic data production, libraries will be constructed for each section of the feeding tube at the Interdisciplinary Center for Biotechnology Research at the University of Florida (ICBR). Libraries will be sequenced on the Illumina HiSeq 2500 platform, targeting ~3 Gb of sequence per sample with 100 bp, paired-end reads. Profiling of metabolic pathways will be performed using with Blast2GO at https://www.blast2go.com. To identify genes encoding proteins with potential antibiotic resistance functions, the metagenomes will be analyzed using ResFinder 2.1. The ResFinder program (http://cge.cbs.dtu.dk/services/ResFinder/) is an acquired antimicrobial resistance search tool which identifies genes with > 40% identity to known antibiotic resistance genes to identify genes encoding proteins with potential virulence functions will be used VirulenceFinder 1.5 (http://cge.cbs.dtu.dk/services/VirulenceFinder/). Using this tool curated by the Center for Genomic Epidemiology we will be able to identify virulence factors associated with adherence,

toxin production, exoenzymes, host immune evasion, and secretion. Based on the findings of the metagenomic data, the antibiotic susceptibility of bacterial isolates will be confirmed by antimicrobial susceptibility tests using the Kirby-Bauer method.
To determine bacteria in the feeding tube which may originate from the mother's breast milk or donor milk, a small sample will be obtained at week 1 and week 4 and similar 16S rRNA and metagenomics sequencing will be performed. If still providing breast milk to their infant, the infant's mother will be asked to provide 2-5 ml of her breast milk which she has pumped per standard NICU policy. This sample will be analyzed using similar microbiome and metagenomic analyses.

Biofilm Analysis. The presence of biofilm in the interior surface of the feeding tubes will be determined using fluorescent stains and visualized by confocal microscopy. We will utilize a variety of fluorophores to discriminate live/dead cells (FilmTracer LIVE/DEAD biofilm viability kit, Invitrogen) and the matrix components (FilmTracer SYPRO/Ruby biofilm matrix stain, Invitrogen) as directed by the manufacturer. The results obtained will be confirmed by scanning electron microscopy (SEM).

Gastric/Stool/Sputum/Sampling. Gastric sample of at least 1.0-2.0 mL will be collected weekly by the research coordinator, immediately following insertion of a new feeding tube, by gently aspirating gastric contents from the feeding tube into a syringe. Gastric aspiration assessment for feeding tube placement as per NICU protocol. One baseline pH will be obtained at start of study. To assess gastric pH, the research coordinator will use one-two drops of the gastric sample to test using Hydrion pH strips and Fischer Scientific Horiba LAQUA twin pH 11 meter. The rest of the gastric sample will be used to analyze the microbiome and level of inflammatory markers. Stool samples will be collected weekly by the research nurse and pH check of sample will be done. Following collection, all samples will be placed in a sterile specimen container and immediately frozen at -80 degrees Celsius for later analysis. This team has extensive experience in the collection of gastric aspirates, and stool from premature VLBW infants in the NICU as well as the analysis of inflammatory markers and 16S rRNA sequencing of these samples. Oropharyngeal swabs will be collected weekly by the research coordinator (a registered nurse experienced in the care of critically ill infants) by placing a tiny soft brush into the infant's mouth and gently rotating the swab to collect the infant's saliva.

Microbiota Sequencing and Analyses: DNA from samples will be extracted and analyzed as described above (see Aim 1).

Inflammatory Response. The samples will be analyzed for inflammatory markers using a combination of multiplex technologies using the BioRad Bio-Plex platform as well as S100A12 and calprotectin immunoassays.

Statistical Analysis

Data integrity will be evaluated using descriptive statistics (e.g.: means, standard deviations, frequencies, percents, range) appropriate for measurement level. Checks for implausible or out-of-range values, distributional forms, the presence and distribution of censored values (e.g.: inflammatory markers), and missingness will be performed. Data transformations (e.g.: Box-Cox family of transforms) will be made, if required, based on evaluation of model fit and tenability of assumptions. If applicable, regression techniques which can accommodate censored variables (e.g. MPLUS version 8 or higher)[58] will be employed. Selected variables (e.g.: type of feed (mother's breast milk or donor breast milk), whether NPO, antibiotic use, gestational age, chronological age, gastric pH and sex) will be screened for potential inclusion as covariates in each of the statistical models tested. Associations between each potential covariate and the respective dependent variable will be examined, and only those variables having a $p<.10$

association and whose inclusion improves model fit will be retained. The general modeling strategy will be a generalized linear mixed model (GLMM) approach. A GLMM is able to accommodate different numbers of measurements between subjects, utilize data from subjects when some of the data is missing (that is, subjects with some missing data points will not be dropped from analysis), can incorporate time-varying covariates, flexible covariance structures, and a variety of dependent variable distributions (including dichotomous), and thus will be able to accommodate the analyses proposed. Model fit and strength of association will be evaluated using Vonesh's GOF macro for SAS. Our primary goal will be to assess differences in marginal least squares means between the dwell time groups. All analyses will be performed using SAS software version 9.4 or later. Statistically significant interaction effects will be evaluated using simple main effects analyses via the SLICE option available in the SAS/STAT GLMM procedures. Due to the number of inferential tests planned, it is not practical to maintain a 5% experiment-wise Type I error rate; however, significance levels will be adjusted to maintain a model-wise 5% Type I error rate through use of a Benjamini-Hochberg approach.

Data Entry and Management

A code key stored separate from the data set will be used. The code key will be kept in a locked cabinet in the research office. All data will be entered into a REDCAP database having integrated data quality and consistency checks. The study database created in REDCap will be password-protected and housed on a designated database server. Data quality will be monitored and assured as reported and as entered into the database. All hardcopy forms will be

Variable	Measurement	Timing
Descriptive (1-2) below		
1. Maternal, prenatal and perinatal demographics	Maternal history, prenatal and perinatal complications and medications, mode of delivery, *antibiotic use, steroid use*	Collected upon entry into study.
2. Infant demographics	Race/ethnicity, sex, gestational age, birth weight, Apgar scores, resuscitation at birth and neonatal acuity (SNAP II) score, *type of feeding, whether or not the infant is NPO, medications effecting gastric pH, chronologic age, NPO, number of days and type of antibiotics*	Collected upon entry into study and during the *4* week study period. Antibiotic data tracked for 6 weeks
Microbial analysis of feeding tubes, feeding tube hubs and residual intraluminal liquid	Culture based analysis and microbial analysis (*16S rRNA and metagenomics sequencing).* Presence of biofilm in feeding tube or Replogle tube	Following removal of each feeding tube for *4* weeks
Analysis of gastric contents (1-2) below		
1. Microbial analysis	*16S rRNA and metagenomics sequencing*	Weekly for *4* weeks
2. Inflammatory markers	Analysis of inflammatory markers	Weekly for *4* weeks
3. pH testing	Measuring acidity	Initially and weekly for 4 weeks
Analysis of stool (1-2) below		
1. Microbial analysis	*16S rRNA and metagenomics sequencing*	Weekly for *4* weeks
2. Inflammatory markers	Analysis of inflammatory markers	Weekly for *4* weeks
3. pH testing	Measuring acidity	Weekly for 4 weeks
Nutritional outcomes (1-6) below		
1. Enteral intake	24-hour enteral feeding intake in mL/kg	Daily for *6* weeks
2. Time to full feeds	First day infant received ≥120 mL/kg/d of enteral feedings. First day infant received 150ml/kg/d	Recorded daily until 150 mL/kg/d reached (up to 6 weeks)
3. Hours of PN	Number of hours infant received some parenteral nutrition	Daily for *up to 20 weeks*
1. PNALD	Liver function tests (level of direct bilirubin, alkaline phosphatase, AST and ALT)	Weekly or biweekly for 6 weeks Peak alkaline phosphatase and Peak direct bilirubin for up to 20 weeks or discharge
2. Hours of central venous line access	Number of hours infant has a central venous line	Daily for up to *20 weeks*
3. Growth indices	Weight, length, and head circumference	Daily for *4* weeks and at discharge
4. Episodes of late onset sepsis	Episodes of culture proven or presumed sepsis (treated with ≥3 days of antibiotics but with negative cultures)	Daily from birth up to 20 weeks
5. Episodes of NEC	Episodes of radiologic or surgical evidence of NEC	All incidences for up to 20 weeks
6. Length of hospital stay	Days infant remains in hospital	From birth until discharge
Covariates (1-5) below		
1. Gastric pH	*pH of gastric aspirates, use of medications which affect gastric pH*	*Gastric pH analysis will be done weekly, information regarding medications will be collected during the 4 weeks study period*
2. Oropharyngeal swabs	*Time of last feed, oral medications*	*Sputum samples weekly for 4 weeks*
3. Analysis of maternal breast milk or donor milk	*Microbiota and metagenomic sequencing*	*At week 1 and week 4*

4.Nutritional data	*Medications and disease processes associated with decreased intestinal motility, episodes and duration of kangaroo care*	*Upon entry into study and for 4 active weeks.*

visually inspected before data entry. A manual comparison of randomly selected data hardcopy forms with data output listing generated from the study database will be performed, and consistency checks will be generated by SQL or SAS programs as part of data cleaning procedures. The system will assign a global unique identifier to each enrolled subject that will serve as the subject id. Study case report forms (CRFs) will be maintained in study specific folders and when CRFs not actively being processed, will be secured in locked file cabinets. In addition to the use of passwords and other security measures, all documents containing identifying information are considered confidential materials and will be safeguarded. Data to be collected are found in Table 3. Data collected in REDCap will be shared with an outside affiliated researcher at Old Dominion University for secondary data analysis. All data sets shared will be verified with CTSI REDCap, before giving outside researcher access.

7. Possible Discomforts and Risks:

Although little information exists concerning the discomfort associated with feeding tube replacement in VLBW infants, it is likely associated with some degree of discomfort. Therefore, prior to insertion of each feeding tube, strategies known to reduce pain in neonates will be implemented. Comfort measures are routinely utilized during feeding tube removal and insertion in this NICU. Comfort measures implemented may include the use of a pacifier dipped in a sweet solution for infants with a sustained suck and greater than 28 weeks gestation, (2) 0.2 ml of a sweet solution placed on the gums for infants that are greater than 28 weeks but too ill or immature to have a sustained suck, (3) all infant will be swaddling and/or tucking and (4) use of a securement device or taping method approved by the NICU to minimize skin irritation. These measures are consistent with current practice in this NICU for pain reduction and will include the following; (1) providing a pacifier dipped in a 25% sucrose solution for infants who have a sustained suck, (2) placing 0.2 ml of a 25% sucrose solution in the oral/buccal cavity for infants too immature or ill to have a sustained suck and (3) provide all infants with swaddling and facilitated tucking. Sucrose solutions given orally before painful procedures has been extensively studied are safe, well tolerated and reduce pain in premature VLBW infants.

8. Possible Benefits:

Feeding tubes that remain in place for a shorter period of time may be less contaminated and lead to decreased complications