Published in final edited form as:

J Pediatr Surg. 2011 June ; 46(6): 1029–1033. doi:10.1016/j.jpedsurg.2011.03.026.

Bacterial DNA Content in the Intestinal Wall from Infants with Necrotizing Enterocolitis

Brian T. Bucher¹, Lucas A. McDuffie¹, Nurmohammad Shaikh², Phillip I. Tarr², Barbara B. Warner³, Aaron Hamvas³, Francis V. White⁴, Christopher R. Erwin¹, and Brad W. Warner¹

¹Division of Pediatric Surgery, Department of Surgery Washington University School of Medicine, St. Louis, MO

²Division of Gastroenterology Washington University School of Medicine, St. Louis, MO

³Newborn Medicine, Department of Pediatrics Washington University School of Medicine, St. Louis, MO

⁴Department of Pathology and Immunology; Washington University School of Medicine, St. Louis, MO

Abstract

PURPOSE—The objectives of our study were to quantify mucosal bacterial DNA within specimens from neonates undergoing small bowel resection for necrotizing enterocolitis (NEC).

METHODS—We obtained clinical information and pathologic specimens from all infants diagnosed with NEC who underwent surgical treatment at our institution from 1999–2008. Bacterial and human DNA were isolated from paraffin-embedded surgical specimens and real-time PCR was used to amplify bacterial and human genes. Linear regression was used to quantify the amount of human and bacterial DNA in our specimens.

RESULTS—From a cohort of fifty infants, we identified twenty-three infants who underwent both surgical resection and subsequent intestinal reanastomosis. Thirteen (59%) of the neonates had Bell's Stage III NEC, and nine (41%) had Stage II. There was significantly more bacterial DNA in the resection specimens than in the reanastomosis specimens. This corresponds to a median (IQR) increase of 1.81 (1.11–4.69) fold bacterial DNA in the resection specimen compared to the reanastomosis specimen ($p < 0.05$).

CONCLUSION—There is more bacterial DNA in infants with acute NEC compared with the same infants after the NEC had clinically resolved. These findings underscore the potential relevance of adherent or invasive bacteria across the bowel wall in the pathogenesis of NEC.

Keywords

Necrotizing Enterocolitis; 16S DNA; Bacteria Translocation; Metagenomics

© 2011 Elsevier Inc. All rights reserved

Corresponding Author: Brian T. Bucher, MD Department of Surgery Washington University School of Medicine 660 South Euclid Ave Box 8235 St. Louis, MO 63110 Phone (314) 454-6022 Fax (314) 454-4224 bucherb@wudosis.wustl.edu. Request for Reprints: Brad W. Warner, MD Division of Pediatric Surgery Department of Surgery Washington University School of Medicine 660 South Euclid Ave Box 8235 St. Louis, MO 63110 Phone (314) 454-6022 Fax (314) 454-4224 brad.warner@wustl.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

The authors have no financial disclosures.

INTRODUCTION

Necrotizing enterocolitis (NEC) continues to be a significant cause of morbidity and mortality for premature infants. [1] Because of significant advances in the care of the preterm infant, the number of infants at risk for NEC continues to increase as mortality from respiratory failure has declined. [2] NEC is now the chief cause of mortality in premature infants, with in-hospital case fatality rates for this disorder ranging from 15 to 30%, and approaches 100% in the most severe form of the disease, NEC totalis. [3–5] The survivors of NEC are at increased risk for additional morbidities including short bowel syndrome, TPN-induced cholestasis, and developmental delay. [6–8]

The pathogenesis of NEC was first described in 1975 by Santulli who noted three factors that are important for the development of NEC: injury to the intestinal mucosa, presence of intestinal bacteria, and feedings. [9] Since that time numerous studies have attempted to identify the host, microbiologic, and environmental factors related to the development of this disease. A number of host risk factors, including a genetic predisposition, premature birth, and enteral feeding status have been implicated in the disease pathophysiology of NEC. [10–12] The role of the intestinal bacteria in the development of NEC is now gaining greater attention.

Most efforts to identify microbial correlates of NEC have been limited to stool culture-based investigations obtained from patients with or at risk for NEC. Indeed, several bacterial species including *Clostridium* spp., *Klebsiella* spp., *Escherichia coli*, *Enterobacter* spp. and others have been linked to disease pathogenesis. [13, 14] Recently with the advances in high throughput DNA sequencing, culture-independent based methodology based on sequencing of the 16S ribosome gene DNA has expanded the ability study bacterial communities in the human intestine. [15] Several studies have used the above methodology to identify causative organisms obtained from stool samples of infants with NEC with limited success. [16] The analysis of stool samples, however, is inherently limited to the study of the non-adherent bacteria in the gut. No studies have used metagenomic sequencing to identify the organisms involved in the bacterial invasion associated with NEC.

The objectives of the current study were to determine if bacterial DNA could be successfully extracted and analyzed from pathologic specimens of infants with NEC. Secondly, we attempted to quantify the degree of bacterial invasion in infants with acute NEC compared to the same infants with the NEC had clinically resolved.

1. METHODS

1.1 Patient Population

St. Louis Children's Hospital (SLCH) is a 235-bed academic tertiary care center affiliated with Washington University School of Medicine. We retrospectively identified all patients admitted to SLCH from January 1, 1999 to June 30, 2009 who had both a diagnosis of NEC, based on the International Classification of Diseases diagnosis code of 777.5, and underwent intestinal resection. Patients were included in our study if they had a clinical diagnosis of necrotizing enterocolitis, and underwent both an intestinal resection with fecal diversion followed by a subsequent intestinal reanastomosis. Patients were excluded from our study if they did not have a clinical diagnosis of NEC, had no pathologic specimens available, did not undergo both a small bowel resection and a subsequent reanastomosis, or did not have intact tissue appropriate for DNA isolation. Institutional Review Board approval was obtained to perform this research from the Washington University Human Research Protection Office.

1.2 Patient Data

The medical records of all included children were retrospectively reviewed to abstract pertinent clinical information. Data abstracted included patient demographics, prenatal course and birth history, congenital comorbidities, risk factors for NEC, date of NEC diagnosis, Bell's stage of NEC [17], indications for surgical interventions, location and length of resection, and date of intestinal reanastomosis. Outcome variables included survival to reanastomosis.

1.3 Bacterial Quantification

The pathologic specimens for all included patients were obtained from the Washington University Department of Pathology. For each specimen, DNA was extracted from paraffin blocks using the Gentra Puregene Tissue Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Each specimen's DNA sample was then used for quantitative real-time PCR for both bacteria 16S ribosomal DNA (rDNA) and human GAPDH genes representing the bacteria and host respectively. Custom primers were designed for both bacterial 16S ribosomal DNA (Forward 5' AAC TCC TAC GGG AGG CAG CAG 3', Reverse 5' AAG GAC TAC CAG GGT ATC TAA TCC 3') and human GAPDH (Forward 5' ACA ACG AAT TTG GCT ACA GC 3', Reverse 5' TGA TGG TAC ATG ACA AGG TGC 3'). SYBR Advantage qPCR Premix (Clontech, Mountain View CA) and an Applied Biosystems 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City CA) were used to amplify the PCR products of each gene. Standard curves for each gene product were constructed using known quantities of purified human and *Escherichia coli* DNA. For each specimen the quantitative PCR was done in triplicate and the quantity of bacterial DNA was normalized to the amount of human DNA.

1.4 Statistical Analysis

Data are presented as medians, with interquartile ranges (IQR) in parentheses. Standard curves were constructed using linear regression analysis. The quantities of both human and bacterial DNA in each specimen were extrapolated from the standard curves of each gene product. The Wilcoxon signed rank nonparametric test was used to determine statistical significance, with a P value < 0.05 considered significant. Data analysis was performed using Microsoft Excel (Microsoft Corp, Redmond, WA) and GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA).

2. RESULTS

From a cohort of 50 infants who had a diagnosis of NEC and underwent an intestinal resection, we identified 23 (46%) infants who met the inclusion criteria. The patient demographics, clinical course and outcomes are shown in Table 1. The median gestational age and birth weight was 29 (25.7–33.1) weeks and 1220 (760–2178) grams, respectively. The median post conceptual age at the time of NEC diagnosis was 32.4 (29.9–36.4) weeks. Most (78%) of infants with NEC had pneumatosis intestinalis. The majority of infants were either Bells Stage II (30%) or III (65%). A median of 17 (8–24) centimeters of small intestine was resected from each patient. The median time between the resection and the reanastomosis was 10 (8.7–11.7) weeks. Overall, 78% of the patients survived long enough to undergo intestinal reanastomosis.

We next used quantitative PCR to quantify the amount of bacteria rDNA present in the specimens obtain from patient both at the time of intestinal resection for NEC and the subsequent intestinal reanastomosis (Figure 1). After normalizing to the amount of human DNA in each specimen, there was significantly more bacterial rDNA in the resection specimen than in the intestinal reanastomosis specimen (median bacteria [ng]/human [μg]

DNA ratio: 4.57 vs. 4.10, $p < 0.05$). This corresponds to a median fold increase of 1.8 (1.1–4.7) of bacterial rDNA in the resection specimen compared to the reanastomosis specimen.

There were no significant correlations between the any of the clinical parameters presented in Table 1, including the patient's Bell's stage, and the amount of bacteria present in the resection specimen. In addition there were no significant correlations between the increase in bacterial amount in the specimens and any of the clinical parameters in Table 1.

3. DISCUSSION

The purpose of the current study was to determine if bacterial DNA could be successfully extracted and analyzed from pathologic specimens of infants with NEC. We identified 23 infants who had both an intestinal resection specimen and a subsequent reanastomosis specimen. Using standard DNA isolation techniques, we were able to obtain bacterial DNA from pathologic specimens up to 10 years old. Our second objective was to quantify the degree of bacterial invasion in infants with acute NEC compared to the same infants with the NEC had clinically resolved. The patient cohort used in this study included infants with the most severe form of NEC (Bell's Stage II & III) who underwent intestinal resection during the acute NEC episode. We identified a significant increase in the amount of bacterial 16S rDNA present in the intestine at the time of initial SBR for NEC compared to the same patient at the time of intestinal reanastomosis. There were not significant correlations between any of the clinical parameters and the amount of bacteria present in the resection specimens.

The role of the intestinal bacteria was first linked to the pathophysiology of NEC by Santulli in 1975 who noted the three necessary factors for the development of NEC: injury to the intestinal mucosa, presence of intestinal bacteria, and feedings. [9] Our results showing increase amount of tissue bacteria at the time of intestinal resection for NEC again highlight the role of the intestinal bacteria in the pathogenesis of this disease. Several bacterial species including *Clostridium*, *Klebsiell.*, *Escherichia coli*, *Enterobacter* and others have been linked to disease pathogenesis. [13] Our findings would support the role of invasive bacteria in the pathogenesis of NEC as we identified an approximately two fold increase in the amount of bacterial 16s rDNA present at the time of acute NEC. The increase in bacterial DNA at the time of NEC can occur by either bacterial overgrowth or by destruction of normal human tissue. The current study cannot distinguish which of these processes are occurring.

Several studies have used 16S rDNA sequencing to identify the role of intestinal bacterial in the pathogenesis of NEC. De la Cochetiere studied 12 neonates, 3 of whom developed NEC, and described colonization by *Clostridium perfringens* prior to the development of NEC. [18] Wang studied 10 infants with NEC along with 10 controls and noted a low bacterial diversity in the 10 infants who developed NEC. [19] The majority of these studies suffer from small sample sizes and the limitation of using bacterial obtained from stool samples. In our present study, which we did not perform 16s rDNA sequencing, our sample were taken from the pathologic tissue specimens not the GI luminal contents. This highlights the role of adherent or invasive bacteria in the pathophysiology of NEC.

Our study does have the limitations associated with any retrospective study including lack of standardization with tissue collection and processing. This lack of standardization may lead to luminal contamination of the pathologic specimens. The use of previously preserved paraffin imbedded tissue was made due to its availability. It is possible that the fixation and storage of these specimens may have altered to bacterial content. Future studies using freshly frozen tissue will account for this limitation. We elected to use the infants as their own controls by comparing specimens obtain at the time of resection for NEC versus after

the NEC had clinically resolved. Other potential control groups that could have been used include infants undergoing intestinal resection for non-NEC indications. However the use of these infants as controls is confounded by differences in age, degree of prematurity, antibiotic exposure, and baseline bacterial content differences. We did not microscopically identify any luminal contents in the specimens; however, this finding does not exclude the possibility of luminal contamination. There is also the possibility that the 16s rDNA genes detected represent contamination. However, we attempted to limit this by processing the paired specimens (resection and reanastomosis) together, thus limiting differential contamination from processing.

4. CONCLUSION

In this study, we were able to obtain DNA from pathologic tissues specimens of infants with NEC. There was significantly more bacterial DNA in infants with acute NEC compared with the same infants after the NEC had clinically resolved. These findings underscore the potential relevance of translocation of luminal bacteria across the bowel wall in the pathogenesis of NEC.

Acknowledgments

This work was funded in part through the following grants from the National Institutes of Health: NIH 5T32GM00879509 (BTB) and 1UH2AI083265-01 (PIT, BBW) and the St. Louis Children's Hospital Foundation – Children's Surgical Sciences Research Institute.

REFERENCES

1. Guner YS, Chokshi N, Petrosyan M, et al. Necrotizing enterocolitis--bench to bedside: novel and emerging strategies. *Semin Pediatr Surg.* 2008; 17:255–265. [PubMed: 19019294]
2. Holman RC, Stoll BJ, Clarke MJ, et al. The epidemiology of necrotizing enterocolitis infant mortality in the United States. *Am J Public Health.* 1997; 87:2026–2031. [PubMed: 9431297]
3. Abdullah F, Zhang Y, Camp M, et al. Necrotizing enterocolitis in 20,822 infants: analysis of medical and surgical treatments. *Clin Pediatr (Phila).* 2010; 49:166–171. [PubMed: 20080523]
4. Holman RC, Stoll BJ, Curns AT, et al. Necrotising enterocolitis hospitalisations among neonates in the United States. *Paediatr Perinat Epidemiol.* 2006; 20:498–506. [PubMed: 17052286]
5. Ricketts RR, Jerles ML. Neonatal necrotizing enterocolitis: experience with 100 consecutive surgical patients. *World J Surg.* 1990; 14:600–605. [PubMed: 2238659]
6. Horwitz JR, Lally KP, Cheu HW, et al. Complications after surgical intervention for necrotizing enterocolitis: a multicenter review. *J Pediatr Surg.* 1995; 30:994–998. discussion 998–999. [PubMed: 7472960]
7. Moss RL, Das JB, Raffensperger JG. Necrotizing enterocolitis and total parenteral nutrition-associated cholestasis. *Nutrition.* 1996; 12:340–343. [PubMed: 8875518]
8. Yeh TC, Chang JH, Kao HA, et al. Necrotizing enterocolitis in infants: clinical outcome and influence on growth and neurodevelopment. *J Formos Med Assoc.* 2004; 103:761–766. [PubMed: 15490026]
9. Santulli TV, Schullinger JN, Heird WC, et al. Acute necrotizing enterocolitis in infancy: a review of 64 cases. *Pediatrics.* 1975; 55:376–387. [PubMed: 1143976]
10. Henderson G, Craig S, Baier RJ, et al. Cytokine gene polymorphisms in preterm infants with necrotising enterocolitis: genetic association study. *Arch Dis Child Fetal Neonatal Ed.* 2009; 94:F124–128. [PubMed: 17768156]
11. Neu J, Chen M, Beierle E. Intestinal innate immunity: how does it relate to the pathogenesis of necrotizing enterocolitis. *Semin Pediatr Surg.* 2005; 14:137–144. [PubMed: 16084400]
12. Warner BW, Warner BB. Role of epidermal growth factor in the pathogenesis of neonatal necrotizing enterocolitis. *Semin Pediatr Surg.* 2005; 14:175–180. [PubMed: 16084405]

13. Morowitz MJ, Poroyko V, Caplan M, et al. Redefining the role of intestinal microbes in the pathogenesis of necrotizing enterocolitis. *Pediatrics*. 2010; 125:777–785. [PubMed: 20308210]
14. Bell MJ, Shackelford PG, Feigin RD, et al. Alterations in gastrointestinal microflora during antimicrobial therapy for necrotizing enterocolitis. *Pediatrics*. 1979; 63:425–428. [PubMed: 440844]
15. Ley RE, Lozupone CA, Hamady M, et al. Worlds within worlds: evolution of the vertebrate gut microbiota. *Nat Rev Microbiol*. 2008; 6:776–788. [PubMed: 18794915]
16. Mshvildadze M, Neu J, Shuster J, et al. Intestinal microbial ecology in premature infants assessed with non-culture-based techniques. *J Pediatr*. 2009; 156:20–25. [PubMed: 19783002]
17. Bell MJ, Ternberg JL, Feigin RD, et al. Neonatal necrotizing enterocolitis. Therapeutic decisions based upon clinical staging. *Ann Surg*. 1978; 187:1–7.
18. de la Cochetiere MF, Piloquet H, des Robert C, et al. Early intestinal bacterial colonization and necrotizing enterocolitis in premature infants: the putative role of *Clostridium*. *Pediatr Res*. 2004; 56:366–370. [PubMed: 15201403]
19. Wang Y, Hoenig JD, Malin KJ, et al. 16S rRNA gene-based analysis of fecal microbiota from preterm infants with and without necrotizing enterocolitis. *ISME J*. 2009; 3:944–954. [PubMed: 19369970]

Figure 1.
Ratio of bacteria to human DNA in the intestinal resection and reanastomosis specimens.
(n=23, *p<0.05).

Table 1

Clinical characteristics of patients included in this study.

Total	23
Demographics	
Male ¹	11 (48%)
Race ¹	
Caucasian	10 (44%)
African American	11 (48%)
Other	2 (9%)
Gestational age ² in weeks	29.0 (25.7–33.1)
Birthweight ² in grams	1220 (760–2178)
NEC Diagnosis	
Post Conceptual Age at Diagnosis ² in weeks	32.4 (29.9–36.4)
Abdominal Radiographic Findings ¹	
Pneumatosis intestinalis	18 (78%)
Portal Venous Gas	8 (35%)
Free Intraperitoneal Air	11 (47%)
Bells Stage ¹	
I	1 (4%)
II	7 (30%)
III	15 (65%)
Length of Intestinal Resection ² in cm	17 (8–24)
Type of Intestine Resected ¹	
Jejunum	4 (17%)
Jejunum and Ileum	6 (26%)
Ileum	7 (30%)
Ileum and Colon	6 (26%)
Outcomes¹	
Survival	18 (78%)

¹Value represents total number (percentage).²Value represents median (IQR).