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Absence of Gastrointestinal Pathogens in Ileum Tissue Resected for Necrotizing Enterocolitis

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Abstract

Necrotizing enterocolitis (NEC) is the one of the most common gastrointestinal emergencies in premature infants and has been linked with viral antigens for as much as 40% of cases in single center cohorts. We examined 28 tissue sections from surgically resected ileum from 27 preterm infants with NEC from 2 separate institutions for 15 common bacterial, viral, and parasitic gastrointestinal pathogens using multiplex RT-PCR amplification and suspension array detection methods. We did not detect infectious enteritis pathogens in any of the NEC tissues and conclude that gastrointestinal pathogens are a rare cause of NEC.

Keywords

Necrotizing Enterocolitis; Intestinal Diseases; Reverse Transcriptase Polymerase Chain Reaction

Introduction

Necrotizing enterocolitis (NEC) is a complex, multifactorial complication of prematurity characterized by an acute inflammatory cascade leading to bowel necrosis. It is the most common acquired gastrointestinal medical and surgical emergency in premature infants and occurs in 7% of infants less than 1500 g birth weight[1,2]. Progress in prevention of the disease has been limited by the inclusion of heterogeneous intestinal diseases labeled as NEC[3]. Although considered an infectious disease for a long time, no single pathogen has been identified consistently. Reports of NEC have linked this disease with a variety of intestinal pathogens including *Clostridium difficile*[4], *Clostridium perfringens*[5], *Escherichia coli*[6], adenovirus[7–8], astrovirus[9], enterovirus[10], rotavirus[11], and norovirus[12]. The number of reports of viral pathogens associated with NEC has tripled in the last 5 years and it has been suggested that rotavirus may constitute as much as 30% and norovirus 40% of NEC cases in

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single center cohorts[3,11–12]. Recently molecular methods have been developed for detection of viral RNA in formalin-fixed, paraffin-embedded human tissues[13]. We applied a multiplex PCR amplification and suspension array detection approach to evaluate 28 ileum tissue samples resected for NEC collected over a 3-year period for 15 common bacterial, fungal and viral gastrointestinal pathogens.

Material and Methods

Clinical Specimens

Fresh ileum tissue specimens from infants with NEC or non-NEC diagnoses were provided from the Pathology departments of the Monroe Carell Jr. Children's Hospital at Vanderbilt and the Children's Hospital of University of Illinois under protocols approved by the respective Institutional Review Boards. All samples were de-identified and only demographic data pertinent to the study design (diagnosis and indication for tissue resection, age at time of tissue resection, gestational age, and sex) were collected from patient records by the pathologist prior to releasing the tissue. Parts of resected tissue were formalin-fixed and paraffin-embedded (FFPE) at the day of tissue collection. All NEC and non-NEC tissue samples were from the small intestine. Positive controls was a paraffin-embedded tissue sample from a pediatric patient with Giardia enteritis and a stool sample from a patient infected with norovirus.

Nuceleic Acid Extraction

Approximately 25 mg of ileum tissue specimen was usedfor total nucleic acid extraction using a NucliSENS easyMAG automated sample preparation system (BioMérieux, Inc., Durham, NC). An off-board process including a frozen tissue grounding or an FFPE tissue paraffin removing was included prior to easyMAG extraction[14]. All extracts were tested positive for human beta-actin DNA and RNA as a quality control approach.

Gastrointestinal Pathogen Detection and Identification

Detection and identification of 15 bacterial (*Salmonella*, *Shigella*, *Campylobacter*, *Clostridium difficile* Toxin A/B, Enterotoxigenic *E. coli* (ETEC) toxins, *E. coli* O157, Shiga-like toxin producing *E. coli* (STEC) stx 1/stx 2, *Vibrio cholerae*, *Yersinia enterocolitica*), protozoal (*Giardia*, *Entamoeba*, *histolytica*, *Cryptosporidium*) and viral (Adenovirus 40/41, Rotavirus A, Norovirus GI/GII) major gastrointestinal pathogens was performed by using an xTAG® Gastrointestinal Pathogen Panel (GPP, Luminex Corp, Austin, TX). Nucleic acid extracts were tested according to the manufacturer's instructions in a 96-well plate format. The Gastrointestinal Pathogen Panel assay comprised a single multiplex PCR with labeled primers, followed by a single-step hybridization of PCR products to the fluorescent bead array and incubation with reporter reagents. The plate was then analyzed using the xMAP 200 IS instrument (Luminex), and the median fluorescent intensity (MFI)was determined[15]. An MFI value above the threshold level determined by the manufacturer for a particular target indicated a positive result for that target.

Results

We analyzed a total of 37 paraffin-embedded human samples. Twenty-three ileum samples were from 22 cases of NEC, 14 samples were non-NEC controls with diverse diagnoses and one sample was from a child with clinical confirmed *Giardia colitis*. Of the NEC samples, 13 were from males and 9 from females (see Table, Supplemental Digital Content 1). The median (range) gestational age was 28.2 (23–39) completed weeks and the median (range) age at the time of tissue resection was 27.1 (7–108) days. All NEC cases were collected between 2007 and 2011 with 2 collected during spring, 5 during summer, 9 during fall and 6

during winter. NEC cases did not appear to occur during a clustered outbreak or a preferential season. Non-NEC controls were taken from patients diagnosed with spontaneous intestinal perforation, atresia, volvulus, ileus or gastroschisis. Of the control samples, 7 were from males and 7 from females. The median (range) gestational age was 32.8 (25–39) completed weeks and the median (range) age at the time of tissue resection was 8.3 (2–73) days. Samples were collected between 2008 and 2011 with 2 collected during spring, 5 during summer, 2 during fall and 5 during winter. The positive control tissue sample was strongly positive for *Giardia* and the stool sample was positive for norovirus. In contrast, none of the NEC or non-NEC tissue samples were positive for any of the following pathogens: *Y. enterocolitica*, *Salmonella*, *Shigella*, *Campylobacter*, *Vibrio*, *E. coli*, *E. coli* ST, *E. coli* LT, norovirus GI, norovirus G II, rotavirus A, adenovirus, *C. diff.* toxin A, *C. diff.* toxin B, *Cryptosporidium*, *E. histolytica*, *Giardia*, Shiga toxin I, and Shiga toxin II.

Discussion

In this study, we did not find any common viral or bacterial pathogen in the collected NEC ileum samples. Several previous studies associated NEC with a variety of epidemic intestinal infections. Recently NEC outbreaks have been linked with norovirus[16–18]. Other studies have linked NEC with *Clostridium perfringens*[5], *Escherichia coli*[6], *Clostridium difficile*[4], rotavirus[11], astrovirus[9] and others. However, in almost all cases pathogen detection has been performed from stool samples but not from tissue. Bhatnagar *et al.* confirmed RT-PCR as highly sensitive method in detection of West Nile Virus in formalin-fixed, paraffin-embedded human tissue samples[13]. Therefore, we applied for the first time a multiplex PCR approach to paraffin-embedded human intestinal tract samples from preterm patients with NEC. To the best of our knowledge this is the largest collection of NEC tissue samples tested for gastrointestinal pathogens by this method.

All affected samples were collected from year-around NEC cases at a stable rate of approximately 20 surgical cases per year in our institution. Reported associations between NEC and bacterial or viral pathogens in previous studies have often been observed as clustered cases simultaneously with epidemic intestinal infections[16–18]. However, *Boccia et al*. could not detect a seasonal trend when they reviewed 17 reports of NEC outbreaks[19]. Other studies have considered a linkage of NEC and viral[9] or bacterial pathogens[5] independent from epidemic infections. The lack of pathogen detection in a relatively large collection of NEC cases supports the multifactorial nature of NEC.

The multiplex PCR approach has its limitations and sensitivity may be too low to detect pathogens with low infectivity. In addition, the assay should be tested in cases of NEC that occurred simultaneously with epidemic bacterial or viral intestinal infections. It's use could be further enhanced if it would cover other commensal anaerobic organisms and be expanded to include further potential pathogens mentioned in the NEC literature such as aeromonas, astrovirus, *Cl. butyricum*, *Cl. perfringens*, coronavirus, coxsackievirus, echovirus, *K. pneumoniae*, and torovirus.

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References

1. Fanaroff AA, et al. Trends in neonatal morbidity and mortality for very low birthweight infants. Am J Obstet Gynecol. 2007; 2:147,e1–8. [PubMed: 17306659]
2. Lin PW, Stoll BJ. Necrotizing enterocolitis. Lancet. 2006; 9543:1271–1283. [PubMed: 17027734]
3. Gordon PV, Swanson JR, Attridge JT, Clark R. Emerging trends in acquired neonatal intestinal disease: is it time to abandon Bell's criteria? J Perinatol. 2007; 11:661–71. [PubMed: 17611610]
4. Cashore WJ, Peter G, Lauermann M, et al. Clostridia colonization and clostridial toxin in neonatal necrotizing enterocolitis. J Pediatr. 1981; 98:308–311. [PubMed: 7463233]
5. Dittmar E, Beyer P, Fischer D, et al. Necrotizing enterocolitis of the neonate with Clostridium perfringens: diagnosis, clinical course, and role of alpha toxin. Eur J Pediatr. 2008; 167:891–895. [PubMed: 17952466]
6. Guner YS, Malhotra A, Ford HR, Stein JE, Kelly LK. Association of Escherichia coli O157:H7 with necrotizing enterocolitis in a full-term infant. Pediatr Surg Int. 2009; 25:459–63. [PubMed: 19396605]
7. Yolken RH, Franklin CC. Gastrointestinal adenovirus: an important cause of morbidity in patients with necrotizing enterocolitis and gastrointestinal surgery. Pediatr Infect Dis. 1985; 4:42–7. [PubMed: 2982133]
8. Cichoki M, Singer G, Beyerlein S, et al. A case of necrotizing enterocolitis associated with adenovirus infection in a term infant with 22q11 deletion syndrome. J Pediatr Surg. 2008; 43:e5–8.
9. Bagci S, Eis-Hübinger AM, Franz AR, et al. Detection of astrovirus in premature infants with necrotizing enterocolitis. Pediatr Infect Dis J. 2008; 27:347–50. [PubMed: 18316982]
10. Birenbaum E, Handsher R, Kuint J, et al. Echovirus type 22 outbreak associated with gastrointestinal disease in a neonatal intensive care unit. Am J Perinatol. 1997; 14:469–73. [PubMed: 9376008]
11. Sharma R, Garrison RD, Tepas JJ III, et al. Rotavirus-associated necrotizing enterocolitis: an insight into a potentially preventable disease? J Pediatr Surg. 2004; 39:453–457. [PubMed: 15017569]
12. Stuart, Boccia D, et al. Nosocomial necrotising enterocolitis outbreaks: epidemiology and control measures. Eur J Pediatr. 2001; 160:385–91. [PubMed: 11421422]
13. Bhatnagar J, Guarner J, Paddock CD, et al. Detection of West Nile virus in formalin-fixed, paraffin-embedded human tissues by RT-PCR: a useful adjunct to conventional tissue-based diagnostic methods. J Clin Virol. 2007; 38:106–11. [PubMed: 17161650]
14. Tang YW. Duplex PCR assay simultaneously detecting and differentiating *Bartonella quintana*, *B. henselae*, and *Coxiella burnetii* in surgical heart valve specimens. J Clin Microbiol. 2009; 47:2647–50. [PubMed: 19553582]
15. Li H, McCormac MA, Estes RW, Sefers SE, Dare RK, Chappell JD, Erdman DD, Wright PF, Tang YW. Simultaneous detection and high-throughput identification of a panel of RNA viruses causing respiratory tract infections. J Clin Microbiol. 2007; 45:2105–9. [PubMed: 17507510]
16. Stuart RL, Tan K, Mahar JE, Kirkwood CD, et al. An outbreak of necrotizing enterocolitis associated with norovirus genotype GII.3. Pediatr Infect Dis J. 2010; 29:644–7. [PubMed: 20589982]
17. Turcios-Ruiz RM, Axelrod P, St John K, et al. Outbreak of necrotizing enterocolitis caused by norovirus in a neonatal intensive care unit. J Pediatr. 2001; 153:339–44. [PubMed: 18534621]
18. Armbrust S, Kramer A, Olbertz D, Zimmermann K, Fusch C. Norovirus infections in preterm infants: wide variety of clinical courses. BMC Res Notes. 2009; 2:96. [PubMed: 19490612]
19. Boccia D, Stolfi I, Lana S, Moro ML. Nosocomial necrotising enterocolitis outbreaks: epidemiology and control measures. Eur J Pediatr. 2001; 160:385–91. [PubMed: 11421422]