The pathophysiology of necrotizing enterocolitis in preterm infants

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CHAPTER 8

IDENTIFICATION OF BACTERIAL INVASION IN NECROTIZING ENTEROCOLITIS SPECIMENS USING FLUORESCENT IN SITU HYBRIDIZATION

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Contents

Abstract

Introduction: We investigated the presence of bacterial invasion together with the identification of bacterial species within the intestinal lumen adhering to and invading into the intestinal wall in necrotizing enterocolitis (NEC) specimens compared to controls.

Methods: We compared 43 surgical NEC specimens (2 sections: least affected and most affected tissue) to age-matched controls (ratio 1:1) derived from intestinal autopsy material. We used fluorescent in situ hybridization (FISH): a universal bacterial probe together with species specific probes for *Clostridium* spp*, Enterobacteriaceae*, bacteroidetes, and enterococci/lactobacilli. We used a FISH scoring system to reveal invasion of the intestinal wall: 1=no/few colonies, 2=average colonies in the intestinal lumen, 3=abundant colonies adhering to the mucosa, and 4=abundant colonies invading the intestinal wall. Bacterial density of each bacterium was determined using the following scoring system: 0=not present, 1=low density, 2=moderate density, 3=high density.

Results: We observed invasion of the intestinal wall in 22/43 of the most affected NEC tissue samples, compared to 16/43 in the least affected NEC tissue samples (p=0.03). A FISH score of 4 was reached in 7/43 control cases. Higher abundances of *Enterobacteriaceae* (density score of 3) were observed in both NEC tissue samples (33/43 respectively 32/43) compared to controls (20/43; both p=0.01). *Clostridium* spp were detected occasionally in NEC samples, bacteroidetes were not observed.

Conclusion: This study demonstrates that invasion of the intestinal wall is more present in most affected NEC tissue samples compared to least affected NEC tissue samples or controls. *Enterobacteriaceae* but not *Clostridium* spp or bacteroidetes are prevalent in advanced NEC.

Introduction

Necrotizing enterocolitis (NEC) is a devastating inflammatory disorder found mostly in preterm infants. NEC characteristically occurs at a postmenstrual age of 30-33 weeks.[1] NEC is characterized by inflammation and necrosis of intestinal tissue. Mortality rates are high (20-30%), and approach 50% in infants who require surgery.[2,3] The underlying pathophysiology of NEC development and progression is still poorly understood.

Amiss bacterial colonization is one of the factors involved in the development of NEC.[2,3] However, the details of this association between NEC development and disease progression remain elusive.[3,4] Previous studies have suggested associations between the presence of microorganisms (such as *Clostridium* spp (including *C. perfringens, C. neonatale, C. butyricum and C. parputrificum*), and NEC development.[4–8] In a prospective study in our center we observed a link between colonization with *C. perfringens* and/or *Bacteroides dorei* and NEC development.[9]

The role of bacterial colonization during NEC progression has not yet been fully elucidated. Via an increase in intestinal wall permeability bacteria may invade the intestine and further aggravate inflammation. In almost all previous studies searching for bacterial colonization processes in NEC, feces samples are used as a surrogate for intestinal tissue. Analysis of feces samples may not be representative because it does not give adequate information on the intestinal microbiota exclusively on the affected site. Few studies focused on the possibility to examine the microbiota at the affected intestinal site – i.e. the intestinal wall. There is no data regarding the different bacterial composition on highly affected versus little affected intestinal sites.

We aimed to investigate the presence of bacterial invasion together with the identification of bacterial species within the intestinal lumen adhering to and invading into the intestinal wall in surgical NEC specimens compared to controls. Using fluorescent in situ hybridization (FISH)[10] we assessed the location and abundances of the bacteria within the intestine (e.g. in the lumen, adhering to the mucosa or invading the intestinal wall) and identified specific bacterial species. We hypothesized that we would observe bacteria invading the intestinal wall more often in more severe NEC cases. Also, we hypothesized that we would observe bacteria commonly associated with NEC (e.g. bacteroidetes, *Clostridium* spp, and *Enterobacteriaceae*) in the severe NEC cases.

Materials & methods

Patients

We performed this retrospective study in a tertiary referral neonatal intensive care unit (NICU) center. The Institutional Review Board of the University Medical Center Groningen approved the study.

We included NEC patients who received surgery between July 2003 and December 2013. Only infants with proven NEC (Bell's stage ≥ 2) who needed surgery were included in the present study. Indications for surgery were bowel perforation (NEC 3b) or lack of improvement despite optimal conservative therapy. The decision to proceed to surgery was always a multidisciplinary decision by the neonatology and pediatric surgery team caring for the infant in combination with elaborate counseling of the parents.

Control patients included preterm infants who died due to cardio respiratory pathology, neurological pathology and/or genetic disorders and underwent an autopsy procedure. Control patients did not suffer from gastrointestinal diseases. We matched controls with NEC patients with regards to gestational age (GA), birth weight, and postmenstrual age (ratio 1:1). We accepted a difference in postmenstrual age of maximal +/- 4 days.

Resection and autopsy specimens

In NEC cases, the pathologist routinely examined the resection specimens and provided the confirmation of the diagnosis. Characteristic macroscopic findings in NEC include a distended bowel, hemorrhage, pneumatosis intestinalis and/or necrosis. Characteristic histological findings in NEC were mucosal edema, hemorrhage, inflammation, transmural bland necrosis, bacterial infiltration and/or collections of gas. Representative sections of normal and diseased bowel were resected and embedded in paraffin. Tissue sections of 4 μm were stained with haematoxylin and eosin (HE) using a standard staining protocol.

We used a previously established histological scoring system for NEC.[11] All samples were assigned a histological NEC severity score (0 – 4) based on the degree of epithelial and/or mucosal damage. When pneumatosis intestinalis and/or necrosis were present, scores of 3 – 4 were given. Both observers (FH/AT) were blinded to the preliminary Bell's stages. We elected two sections of each specimen for further analysis based on the histological scoring: the section with the lowest score (≤2; least affected tissue) and the section with the highest score (≥3; most affected tissue).

In the controls intestinal resection was performed following the general autopsy procedure consisting of macroscopy and microscopy. After general macroscopic pathological examination the intestinal specimens were directly fixated with 10% buffered formalin solution and imbedded in paraffin using standard pathology procedures. Tissue sections of 4μm were stained with HE using the standard staining protocol. As controls for the present study we only included patients in whom the intestinal tissue was considered as non-pathological.

Fluorescent in situ hybridization (FISH) analysis

We used FISH to analyze the location and abundances of bacteria and identify specific bacterial species in the intestinal tissue sections. The sections were deparaffinated by immersion for 2 times in Xylol for 2 min after which the xylol was washed away by immersion for 10 min in 96% ethanol. Deparaffinated slides were dried at ambient air. Bacterial density in the samples was first characterized with a universal bacterial oligonucleotide targeting rRNA probe. We subsequently used the probes as described in Table 1 to detect bacterial populations commonly associated with NEC development.[3,4,8,10] All oligonucleotide probes were obtained from Eurogentec (Seraing, Belgium). For this the slides were hybridized at 50°C overnight in hybridization buffer containing 5 ng μl^{-1} fluorescently-labeled probes. Slides were washed at 50°C in hybridization buffer without sodium dodecyl sulfate (SDS) and mounded with Vectashield® (Vector Laboratories, Burlingame, CA, USA) and a coverslip. We examined the slides using the Leica fluorescence microscope (Leica Microsystems, Wetzlar, Germany).

TABLE 1:

Probes used to analyze the bacterial identity and density in the intestinal sections

Target	Probe	Label	Sequence	Reference
All bacteria	EUB338	Cy3	5'GCTGCCTCCCGTAGGAGT	Amann et al. [12]
Bacteroides/Prevotella#	Bac303	FITC	5'CCAATGTGGGGGACCTT	Manz et al. [13]
Clostridium clusters I and II (*C. perfringens* and relatives)#	Chis0150	FITC	5'TTATGCGGTATTAATCT(T/C) CCTTT	Franks et al. [14]
Enterobacteriaceae#	EC1531	Cy3	5'CACCGTAGTGCCTCGTCATCA	Poulsen et al. [15]
Enterococci/ Lactobacilli *	Lab158	FITC	5'GGTATTAGCA(C/T)CTGTTTCCA	Harmsen et al. [16]

Abbreviations FITC: fluorescein isothiocyanate. Cy3: indocarbocyanine
** Bacterial populations commonly observed in 'healthy' infants*
 # Bacterial populations commonly associated with NEC

Analysis

We investigated the presence of bacterial invasion together with the identification of bacterial species within the intestinal lumen adhering to and invading into intestinal wall in surgical NEC specimens compared to controls using FISH. For analysis we used the most affected section and the least affected section of the NEC samples. We used the FISH score to determine the presence of bacterial invasion, adapted

from Cilieborg et al.[10]: 1=no/few colonies, 2=average colonies in the intestinal lumen, 3=abundant colonies adhering to the mucosa, and 4=abundant colonies invading the bowel wall. We subsequently investigated the density of the separate bacterial species (see Table 1). For both we used the following scoring system: 0=not present, 1=low density, 2=moderate density, 3=high density. During scoring, the assessor was blinded for two categorical groups (NEC versus controls). All scoring was performed within 10 crypts per tissue section (thus 2 scores per NEC case and 1 score for controls).

The $\chi 2$ (chi-square), or Fisher Exact analysis was used for testing differences between categorical variables. For testing differences between two continuous variables the Spearman rho-test was used. To assess differences between the combination of a categorical and a continuous variable the Mann-Whitney *U* test was used. Statistical analyses were performed using the Statistical Package for the Social Sciences (IBM SPSS Statistics 21, IBM Corp., Armonk, New York, USA). All data are presented as median values with range, unless specified otherwise. Two sided p-values less than 0.05 were considered statistically significant.

Results

Patients

We included 43 surgical NEC specimens and 43 controls. Control infants died due to cardio respiratory pathology (28/43; 65%), neurological pathology (8/43; 19%), and/or genetic disorders (7/43; 16%). In the controls no evidence of intestinal disease was present. Age at time of surgery/autopsy was 11 days (4 – 37) for NEC patients and 8 days (2 – 37) for the controls. Patient characteristics are presented in Table 2.

TABLE 2:

Patient characteristics

Patient characteristics	NEC n=43	Controls n=43	P-value
Sex (male)	25 (58%)	27 (63%)	0.66
Gestational age, days	28 (24 – 39)	29 (24 – 39)	0.41
Birth weight, grams	1142 (650 – 2650)	1235 (560 – 2830)	0.52
Caesarean section	16 (37%)	20 (47%)	0.27
Antibiotic therapy post-partum	31 (72%)	37 (86%)	0.10
Antibiotic therapy >48h post-partum	43 (100%)	38 (89%)	0.03*
Enteral feeding post-partum days	0 (0 – 3)	1 (0 – 3)	0.25
Sort of feeding			
- Exclusively mother's milk	2 (4.6%)	2 (4.6%)	0.36
- Exclusively formula	19 (44%)	23 (53%)	0.76
- Both	22 (51%)	18 (42%)	0.43
Age at time of collection of intestinal resection material	11 (4 – 37)	8 (2 – 37)	0,966
NEC Bell's stage 3b	33 (77%)	-	-
Intestinal perforation	26 (60%)	-	-
Removed tissue:			
- Small intestinal	15 (35%)	15 (35%)	0.91
- Small intestinal and large intestine	22 (51%)	22 (51%)	0.91
- Large intestine	6 (14%)	6 (14%)	0.91
Positive blood cultures 48h prior and after date of intestinal resection	3/37 (8.1%)	8/43 (19%)	0,11
Positive peritoneal cultures	18/33 (55%)	-	-
- *Enterobacterieceae*	11/18 (61%)		
- Staphylococci	3/18 (17%)		
- *Clostridium spp*	1/18 (5%)		
- Other	3/18 (17%)		
NICU stay, days (range)	31 (4 – 153)	-	-
Mortality during the acute phase of NEC	12 (28%)	-	-
Overall mortality	16 (37%)	43 (100%)	-

Values are expressed as median (range) if applicable.
*Statistically significant $p < 0.05$

Bacterial invasion

First we used the universal bacterial FISH probe. In 36/43 (84%) of the most affected NEC tissue samples we observed bacteria adhering to the mucosa or invading the intestinal wall (FISH score 3 and 4) compared to 30/43 (70%) in the least affected NEC tissue samples ($p=0.04$). In control cases we observed FISH score 3 and 4 in 8/43 (19%) of the cases (both $p<0.001$ compared to the NEC cases). 22/43 (51%) of the most affected NEC tissue samples scored the maximum FISH score of 4 compared to 16/43 (37%) of the least affected tissue samples ($p=0.03$). A maximum FISH score occurred in 7/43 (16%) of the controls (both $p<0.001$).

Bacterial species

In Figure 1 we present the different bacterial species observed. *Enterobacteriaceae* were most commonly observed. We observed *Enterobacteriaceae* in most affected NEC tissue samples in 33/43 (77%) cases, in least affected NEC tissue samples in 32/43 (74%) cases and in controls in 20/43 (26%) cases. We observed high densities (density score 3) of *Enterobacteriaceae* (Figure 1) in the most and least affected NEC tissue samples (24/33 (73%) and 18/32 (56%) resp.), which were both significantly higher compared to the control tissue samples (7/20 (35%); both $p<0.001$). A density score of 3 of *Enterobacteriaceae* together with a FISH score of 4 in the most and least affected NEC tissue samples was reached in 16/24 (67%) and 7/18 (39%) cases respectively. We detected *Clostridium* spp and enterococci/lactobacilli in 5/43 (12%) respectively 2/43 (4,7%) of the NEC samples. Both were detected only in low numbers (density score 1) and only in the presence of *Enterobacteriaceae*. *Clostridium* spp and enterococci/lactobacilli were not observed in controls. *Bacteroides* were never detected, neither in NEC samples nor in controls.

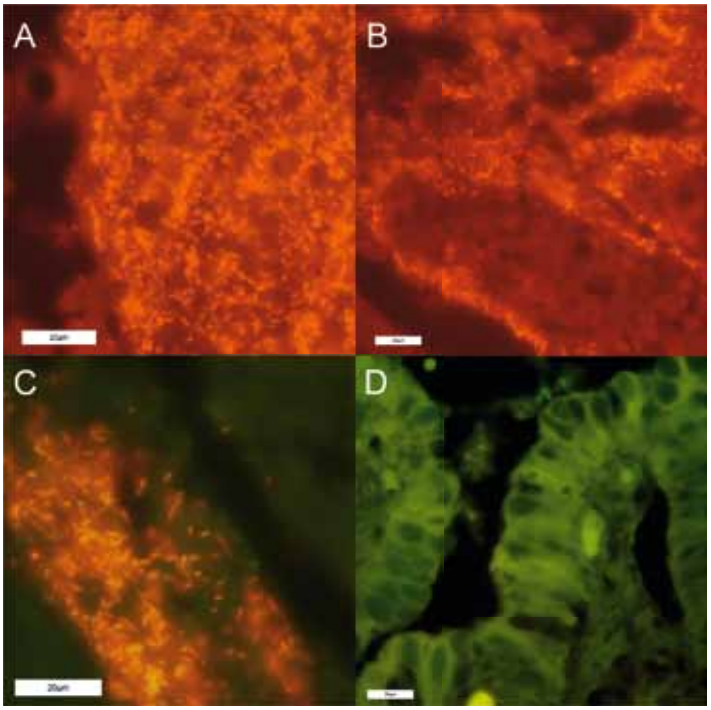

FIGURE 1:

Epifluorescence micrographs of fluorescencent in situ hybridized tissue samples

A: *Bacteria invading the bowel wall (FISH score 4) in a NEC sample hybridized (general bacterial probe (EUB338) tagged with Cy3 (red color)),* ***B:*** *Enterobacteriaeceae*

invading the bowel wall (FISH score 4) in a NEC sample (Enterobacteriaeceae-probe tagged with Cy3) ***C:*** *Clostridium spp (yellow) adhering to the bowel wall (FISH score 3) in a NEC sample (merging of EUB338-probe (tagged with Cy3) and clostridium spp probe (tagged with FITC, green color)).* ***D:*** *Sample with no bacterial colonization (FISH score 1) in the control specimens in the intestinal lumen (EUB338 tagged with FITC). Magnification: 630 - 1000x. The scale bars represent 20 µm in all the micrographs.*

Discussion

This study set out to investigate the presence of bacterial invasion together with the identification of bacterial species within the intestinal lumen adhering to and invading into the intestinal wall in surgical NEC specimens compared to controls. We hypothesized that we would observe bacteria within the intestinal wall more often in more severe NEC cases. Indeed, bacterial invasion in the intestinal wall (FISH score 4) occurred significantly more often in highly affected NEC tissue samples (51%), compared to least affected NEC tissue samples (37%) or controls (16%). This observation suggests that bacterial invasion is associated with the degree of intestinal wall injury. Secondly, in the NEC cases we observed high densities of *Enterobacteriaceae*, while we scarcely observed bacteria commonly associated with NEC development (such as *Clostridium* spp and/or *Bacteroides*). This observation suggests an important role for *Enterobacteriaceae* in the progression of disease.

Higher densities of bacteria were observed in the most affected NEC tissue samples. Our results are in line with three other studies, namely the study of Remon et al.[17] Brower-Sinning, et al.[18], and Smith et al.[19] These three studies also observed proteobacteria (to which *Enterobacteriaceae* belong) in mucosal tissue in NEC specimens. However, these studies were limited by sample size. Our study adds that we differentiated between highly affected NEC tissue samples and least affected NEC tissue samples, and thereby included control samples of patients without (proven) intestinal abnormalities.

While we observed significant more bacterial invasion in highly affected NEC tissue samples (51% of the cases) compared to least affected, in still a noteworthy percentage of the latter bacterial invasion was observed (37% of the cases). This observation suggests that bacterial invasion might be more prevalent during the more advanced stages, but can also be present in less affected tissue. We also detected, surprisingly, bacterial invasion into the intestinal wall in 16% of the control specimens. This finding could be due to the process of dying due to underlying cardio respiratory,

neurological, and/or genetic disorders, even in the absence of pathological findings of the gastro-intestinal tract. We do not believe that bacterial colonization within the intestinal wall occurs in the 'healthy' preterm infant, although literature on this issue is non-existent. Therefore, with our findings it is plausible that bacterial invasion in the intestinal wall can be considered as a complication of the vulnerable preterm intestine, even in infants without evident gastrointestinal abnormalities. Whether this has any pathophysiological consequences is as yet unknown.

Surprisingly, we scarcely observed bacteria associated with NEC development, such as *Clostridium* spp or *Bacteroides*.[8,9,20] McMurtry, et al.[5] reported that abundance of clostridia decreased as the severity of NEC increased. This is in line with the present series – all consisting of severe, i.e. surgical NEC cases. A possible explanation for this observation is the following. *Clostridium* spp (including *C. perfringens*) might decrease gut wall integrity via the production of α-toxins. This in turn could lead to the inflammation leading to NEC.[9,20–22] As a result of the inflammation, oxidative stress is induced by reactive oxygen species (ROS), resulting in an environment in which *Clostridium* spp can hardly survive.[20–22] *Enterobacteriaceae* include versatile species that derive energy for growth from aerobic or anaerobic nitrate respiration or from fermentation.[23,24] *Enterobacteriaceae* are therefore resistant against nutrient variation as well as oxidative stress and can survive in a highly inflamed and necrotic intestine, which is seen during NEC.[25] This phenomenon is also described in the pathogenesis of Crohn's disease, where activated neutrophils infiltrate the intestinal wall and produce ROS, leading to oxidative stress in which *Enterobacteriaceae* survive.[5,11,26] We speculate that *Enterobacteriaceae* invade the bowel wall during NEC development and further aggravate the already present inflammation. The reason that we did not observe lactobacilli is probably because Lactobacilli have only been identified in samples from children with a gestational age greater than 33 weeks and our cohort was significantly younger than that.[27,28]

While we observed high abundances of *Enterobacteriaceae* in the NEC samples, also a fair percentage of the controls were colonized with high densities of *Enterobacteriaceae* (35% with density score 3). We speculate that intensive antibiotic treatment in preterm infants might explain the abundances of *Enterobacteriaceae* in both groups. Tanaka, et al.[29] demonstrated that antibiotics do not clear the intestinal microbiota in infants but reduce the overall diversity of bacterial species. A more intensive antibiotic use reduces the diversity of bacterial species and increase the domination of *Enterobacteriaceae*.[5,30] In a study previously conducted at our center[9] we also observed high abundances of enterobacteriaceae in feces samples during the first days of life in both preterm infants who developed NEC and controls.

Most studies focusing on bacterial colonization during NEC is performed with the use of fecal samples and/or animal experiments. Only three studies analyzed bacterial colonization within the intestine or the intestinal wall.[17–19] In the current study we differentiated between highly affected and least affected tissue of the NEC specimens to be able to study bacterial invasion within the extent of mucosal damage within the same child. We also included a control group matched to gestational age, birth weight, and postmenstrual age. It would be helpful to investigate the bacterial population present in these tissue samples in more detail. Unfortunately, the formalin-fixed paraffin embedded NEC tissues did not allow us to perform 16S sRNA sequencing reliably. Therefore, we were able to assess only a limited selection of bacterial populations. While *Enterobacteriaceae* dominated the specimens, it is possible that we missed other bacterial families that were not assed in the study. Of note, the retrospective nature of this study, in which the intestinal resection material was exposed to oxygen before fixation and was preserved before usage for study purposes, could have influenced our results. Future prospective studies should focus on bacterial identifications and measurement of oxidative stress directly after intestinal resection due to NEC.

In conclusion, we observed bacterial invasion into the intestinal wall in the majority of the most affected NEC tissue samples. Bacterial invasion also occurred in the least affected NEC tissue samples and, to a lesser extent, in the control samples. While we scarcely observed potential bacteria associated with NEC (*Clostridium* spp and *Bacteroides*), we did observe high densities of *Enterobacteriaceae*. While other pathogenic bacteria (such as *C. perfringens* and *B. dorei*) might be associated with NEC development, *Enterobacteriaceae* might have an important role in disease deterioration by invading the intestinal wall and aggravating the inflammation. Further research should investigate the exact relation between invasion with *Enterobacteriaceae* and NEC progression. When indeed, invasion with *Enterobacteriaceae* is linked with NEC progression, there might be an important role for targeted antibiotics during NEC.

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