**Abdulkadir B, Nelson A, Skeath T, Marrs ECL, Perry JD, Cummings SP,
Embleton ND, Berrington JE, Stewart CJ.**

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Stool bacterial load in preterm infants with necrotising enterocolitis

SUMMARY

Resected gut tissue in necrotising enterocolitis (NEC) has a higher bacterial load than controls. Quantitative PCR was performed on longitudinal NEC and control stool samples ($n = 72$). No significant difference in the total bacterial load was found between samples at diagnosis compared to controls or temporally within NEC.

Keywords: Preterm infant; gut microbiome; necrotising enterocolitis; qPCR

Introduction

Necrotising enterocolitis (NEC) is a leading cause of morbidity and mortality in preterm infants in the developed world. The risk of developing NEC increases with lower gestational age and improved survival rates in the most preterm means that NEC is actually increasing. Although bacteria are necessary for NEC, studies have failed to isolate a single causative agent [1]. Therefore, a polymicrobial etiology has been postulated with the diversity of the gut microbiota undergoing significant alterations prior to diagnosis, termed dysbiosis. Thus an emerging theory in NEC research is centred on the total number of bacterial cells, termed the bacterial load, rather than the specific bacterial species in the gut microbiota. A reduced diversity and increased bacterial load in NEC tissue [1] and stool [2] has been found in neonates. Similarly, an increase in mucosa-associated bacterial density was associated with NEC development in preterm pigs [3].

Here we aimed to accurately quantify the bacterial copy number in stool samples from patients with NEC longitudinally in comparison to gestationally matched controls. We hypothesised that total bacterial load, as oppose to abundance of specific species, might provide a useful biomarker for NEC pathogenesis.

Methods

Ethical approval was obtained from County Durham and Tees Valley Research Ethics Committee, and parents gave signed consent for stool and data collection. Detailed patient demographics are presented in supplementary material.

Stool samples ($n = 72$) from 10 infants with NEC ($n = 37$) and 10 matched controls ($n = 35$) were collected from infants cared for in the Royal Victoria Infirmary (Newcastle upon Tyne, UK) and stored at $-80\text{ }^{\circ}\text{C}$. Samples were analysed at -2 weeks (mean -13 days), -1 week (mean -6 days), 0 (mean 0 days), +1 week (mean +6 days), and +2 weeks (mean +15 days),

relative to NEC diagnosis and in comparable samples from matched controls. Matched controls did not differ significantly in **primary** demographics, including birth weight ($P = 0.62$), gestational age ($P = 1.0$), delivery mode ($P = 0.88$), and gender ($P = 0.67$).

DNA was extracted from 100 mg of stool using the PowerLyzer™ PowerSoil® DNA Isolation Kit (MoBio). The plasmid DNA standard was generated and quantitative PCR was performed in triplicate using SYBR green methodology with universal 16S primers (forward 5'-ACTCCTACGGGAGGCAGCAG-3' and reverse 5'- ATTACCGCGGCTGCTGG-3') as previously described [4]. Values were normalised to 100mg of stool (as used in the DNA extraction) and extrapolated to 1g of starting material.

Minitab-16 (State College, PA) was used to generate box plots and **non-parametric Mann-Whitney test** was used for significance testing.

Results and discussion

In 72 stool samples from 10 babies with definite NEC matched individually to 10 controls we saw no association of bacterial load with NEC. With studies failing to isolate causative bacterial strains in NEC, it is important to explore whether the overall bacterial load, regardless of specific species, might be important aetiologically as suggested by previous work [1–3]. We sought to apply comparable quantitative methodology based on molecular sequencing to stool samples, which is a non-invasive means of exploring the gut and clinically more useful mechanism for testing gut health and predicating disease onset.

Our data based on stool showed no significant difference ($P = 0.92$) between NEC and age matched controls at diagnosis, where the total bacterial load per gram of stool was 1.5×10^9 (range $1.4 \times 10^7 - 7.4 \times 10^9$) in NEC and 1.4×10^9 (range $1.4 \times 10^8 - 3.6 \times 10^9$) in controls. **These values are comparable to previous data in preterm neonates [1,2], although notably the copy number increased 10 fold from 10^9 to 10^{10} in samples within 24 hours of NEC diagnosis in**

the study by Jenke *et al.* (2012). Compared to existing data in preterm neonates the . Furthermore, when we considered temporal changes *within* NEC infants prior to and following NEC diagnosis we again found no significant changes in the total bacterial load at each consecutive time point analysed (Figure 1). This was comparable to the temporal data *within* healthy control infants, where no significant changes in the bacterial load occurred in each subsequent week (Figure 1). It has previously been shown in term neonates that the total bacterial load is stable following the first week of life [5], and our infants developed NEC at a median postnatal age of 20 days. Interestingly, Palmer *et al.* (2007) found the copy number per gram in term neonates generally persisted in the range of 10^9 to 10^{10} , an order of magnitude higher than the 10^8 - 10^9 per gram of stool in our infants. Further work is necessary to determine if preterm infants develop a total bacterial load comparable to term infants and to what degree early life intervention, including antibiotics and probiotics, have on this development.

The only significant difference observed was a significant reduction ($P = 0.046$) between the bacterial load of NEC samples and controls 1 week following diagnosis. This may reflect increased antibiotic administration in the NEC infants in the week following diagnosis (median 7 days (average 5 days) in NEC compared to median 0 days (average 1 day) in controls) and ceasing of enteral feeding in the management of NEC. However, 2 weeks after diagnosis the bacterial load of NEC and control infants was again comparable ($P = 0.111$).

Our hypothesis that total bacterial load in stool might provide a useful biomarker for NEC pathogenesis was not found. This is not a reflection of the bacterial load at the site of NEC, rather a non-invasive sample that potentially provides a proxy for gut health. Future studies should aim to compare resected tissue and stool to determine if bacterial load is involved in NEC pathogenesis and if stool provides an accurate means of quantification. Larger cohorts

and more frequent sampling will facilitate sub analysis into the effects of other variables such as gestation, birth mode, and antibiotics.

Conclusion

This study is the first to utilise quantitative PCR to explore the total bacterial load in stool samples from patients with NEC matched to healthy controls. This was not associated with the development of NEC and thus our results suggest that measuring stool bacterial load may not be a proxy measure for tissue bacterial load.

Conflict of interest statement

None declared.

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AUTHORSHIP

CJS, NDE, JEB, SPC and JDP contributed to the study idea and design. BA, CJS, AN, TS, JEB, ECLM, and NDE contributed to collection of samples and acquisition of data. CJS, BA, AN, JEB, NDE, and SPC contributed to analysis of samples and data. All authors have contributed to and reviewed the manuscript. All authors have approved the final article.

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Figure legends

Figure 1 - Total bacterial load between and within NEC and control samples. Horizontal lines inside the boxes represent the median; bars indicate upper and lower quartiles. Asterisks denote significance. Numbers refer to week relative to disease diagnosis. Con – control. NEC – necrotising enterocolitis.