Neonatal Necrotizing Enterocolitis: A Clostridial Disease?

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(See the Major Article by Cassir et al on pages 1107–15.)

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Neonatal necrotizing enterocolitis (NEC) is a devastating disease that remains an important cause of morbidity and mortality among very preterm neonates and whose incidence has increased in parallel with the improved survival of this population. Despite years of research over the past decades, its pathogenesis is still uncertain and thus prevention is difficult. Three factors are recognized to coalesce: immaturity of the preterm infant, enteral feeding, and intestinal bacterial colonization [1]. Although genetics appears to play a role, there is mounting evidence that NEC originates from a defective interaction between intestinal microorganisms and the host’s inflammatory response, leading to the release of mediators and hence to intestinal mucosal injury [2]. However, to date there is no consensus on specific bacteria causally associated with NEC development. Neither culture nor culture-independent approaches have identified a single microbial cause of NEC.

Using culture, various microorganisms have been involved. All belonged to the commensal microbiota and are potential pathogens: Escherichia coli, Klebsiella species, Enterobacter species, Staphylococcus species, and Clostridium species [3]. In particular, the roles of Clostridium perfringens, Clostridium butyricum, and Clostridium neonatale have been reported [4, 5].

During the last decade, culture-independent techniques and, most important, next-generation sequencing (NGS) tools have been applied to microbiota analyses. These tools have allowed identification of bacteria unable to grow under standard laboratory conditions and have expanded our knowledge of microbiota ecosystem composition. Hence, NGS studies have brought new insights in microbiota associated with NEC cases showing an abnormal bacterial pattern and a reduced diversity of microbiota prior to NEC or at the onset of NEC in comparison with controls [6, 7]. However, like culture, different bacterial signatures have been proposed, such as gram-negative bacilli (Enterobacter, Citrobacter, Enterobacteriaceae, or Proteobacteria), Bacillales, or staphylococci. Finally, only a few studies associated NEC with clostridial species colonization [8–10].

The work by Cassir and colleagues, published in the current issue of Clinical Infectious Diseases, investigated the fecal microbiota in infants suffering from NEC compared with control infants. The interest of this study was to use complementary approaches—namely, bacterial culture and 16S ribosomal RNA (rRNA) gene pyrosequencing–based methods. In the first part of their study, through both techniques, the authors found a lower diversity in NEC cases (n = 15) vs controls (n = 15), confirming previously reported results. Moreover, a specific association between NEC and C. butyricum was found in all 15 of the NEC cases colonized by this species compared with only 2 of the control infants. These data were confirmed using a specific quantitative polymerase chain reaction (PCR) assay to screen a larger set of samples of NEC cases (n = 96) vs controls (n = 270). Up to now, this is the first report analyzing fecal samples from infants with NEC by a culture-independent method that identified C. butyricum. Sim et al [9] published a recent study in CID comparing fecal samples from NEC cases (n = 12) vs controls (n = 44) using culture and 16S rRNA gene sequencing methods, and concluded that C. perfringens was significantly overrepresented in NEC cases [9].

Although 16S rRNA gene sequencing is now widely used in microbial ecologic analysis allowing deep analysis of the microbiota, it has its specific limits. First, assignment to specific taxa (family, genus, or species) depends on the length and quality of the sequence. Second, it is based on
PCR amplification using bacterial universal primers or the variable 16S rRNA gene regions, which can lead to missing or underrepresented taxa. Therefore, primer design can be challenging when considering clostridia that belongs to a complex phylogenetic heterogeneous group, with some clusters having high genetic diversity of strains [11]. Moreover, clostridia can be present in a subdominant status, adding to the difficulty of a clear clostridia detection and taxa identification. For example, a culture-based study showed that clostridia (including *C. butyricum* and *C. perfringens*) are part of the commensal microbiota in preterm infants without enteropathies [12]. Cassir and colleagues chose to amplify the V6 region of the 16S rRNA gene, which is not the most frequently used but is presented as better to identify clostridia due to its hypervariability. By contrast, Sim et al amplified the V3–V5 region of the bacterial 16S rRNA genes [9]. This raised the fact that, as already observed concerning bifidobacteria detection in gut microbiota, it will be near-impossible to design a single PCR primer set able to generate an equally efficient amplification yield of 16S rRNA gene sequences across all components of the human gut microbiota [13].

The relationship between *C. butyricum* and NEC is reinforced by the study of Smith and colleagues, who recently reported a correlation between the presence of *C. butyricum* and pneumatosis intestinalis in tissue specimens from neonates with NEC [4]. Moreover, this species was widely involved in studies in the 1980s that specifically isolated by culture *C. butyricum* in blood, peritoneal fluid, or feces from infants with NEC [14–18]. Moreover, its role in NEC pathogenesis has been strengthened in animal models. Indeed, gnotobiotic quails fed a lactose diet and monoassociated with *C. butyricum* display NEC-like digestive lesions [19]. Likewise, a significant role of this species has also been suggested in the preterm piglet model, where *C. butyricum* as well other clostridial species were largely absent in piglets without NEC and significantly overrepresented among colonic mucosal samples from piglets with NEC [20].

Another interesting aspect of Cassir and colleagues’ study is the comparative analysis of culture and 16S rRNA gene sequencing data, which shows only 11% overlap. This demonstrates that, like culture, molecular techniques are far from identifying the totality of fecal microbiota and that complementary approaches as used in this study are therefore necessary. Using bacterial culture allowed Cassir et al to compare the genome of the *C. butyricum* strains for virulence trait screening.

The pathogenesis of NEC can be due to an exaggerated inflammatory response of the immature intestine compared with a mature gut, leading to neutrophil chemotaxis and tissue injury. Various mechanisms have been evoked, for example, increase in proinflammatory cytokine release such as interleukin 8, defect in inhibitors of the nuclear factor (NF-κB) pathway, and activation of Toll-like receptor 4 by lipopolysaccharides from gram-negative bacteria [21]. Some intestinal diseases are associated with bacterial toxins, but this was not the case for NEC. Concerning clostridia, *C. butyricum* bacterial fermentation has been shown to induce NEC-like lesions in the quail NEC model [19], or in rats intracere tally perfused [22]. This is in accordance with the hypothesis of the possible key role of bacteria through overproduction of bacterial fermentation of the nondigested lactose due to intestinal immaturity of the very premature infants [23]. A cytotoxicity effect of *C. butyricum* strain supernatants has been observed in vitro 4 decades ago by means of butyrate [24]. In the current article, Cassir and colleagues report a cytotoxic activity for their 16 *C. butyricum* isolates (including NEC cases and control strains). By sequencing strains’ whole genome, they suggest that cytotoxicity is linked to one gene coding a hemolysin analogue of the etiologic agent of swine dysentery. However, their conclusion must be tempered by the fact that, first, the in silico multilocus sequence type analysis performed to genetically compare *C. butyricum* strains showed clonal relationships between several strains from the same neonatal intensive care unit. The authors did not discuss this aspect. Second, the cytotoxic activity has been found in strains isolated from NEC cases and controls as well. Therefore, the potential role of a putative hemolysin analogue from *C. butyricum* as a toxin that may participate in NEC development needs to be demonstrated.

In conclusion, the manuscript by Cassir and colleagues adds new data regarding the involvement of *Clostridium* in NEC and emphasizes a specific species, *C. butyricum*. It is noteworthy that in other reports NEC was associated with other clostridial species or different separate bacterial signatures (eg, *C. perfringens* and *Klebsiella*) [9].

Up to now, among clostridial species thought to be associated with NEC, none involved known toxin production. By contrast, *C. difficile*, a toxigenic species, is not implicated in NEC [19]. Hence, potential pathogenic characteristics shared by these clostridial species, and perhaps by bacteria belonging to other genera, deserve research. Such characteristics could be specific to the bacterial strains responsible for the disease, as all of these species belong to commensal microbiota, or could interact with the host displaying specific susceptibility.

**Note**

*Potential conflict of interest.* Both authors are involved in the project EPIFLORE, funded by the French National Agency of Research (ANR), which studies the bacterial establishment in very premature neonates and its short- and long-term health consequences.

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