To the Editor—We read with interest the study by Heida et al that aimed to determine, by 16S rRNA Pyrosequencing, the diversity and composition of the intestinal microbiota in preterm neonates at risk for necrotizing enterocolitis (NEC) and its sequential relation to NEC development [1]. In this prospective trial, the authors analyzed the first feces sample and the last 2 feces samples prior to NEC. Notably, the presence and abundance of Clostridium perfringens–like sequences were significantly increased in neonates with NEC compared with controls.

Clostridium Species Identification by 16S rRNA Pyrosequencing Metagenomics

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These findings open promising pathways for the detection and prevention of NEC.

However, we would like to point out some limitations in the interpretation of the specific role of *C. perfringens* in NEC pathogenesis. First, in the study by Heida et al., the V3-V4 region of the 16S rRNA gene was amplified from the bacterial DNA by polymerase chain reaction (PCR) [1]. In another study in which the association between *C. perfringens* and the occurrence of NEC was also reported, the V3-V5 region of the 16S rRNA was amplified [2]. In contrast, in a previous study, we showed that *Clostridium butyricum* was specifically associated with NEC using the V6 region for 16S rRNA pyrosequencing (confirmed by a parallel culture-based analysis and quantitative PCR testing on a larger scale), and another team reported the occurrence of epidemic NEC associated with *Clostridium neonatale* [3, 4]. Such differences could in part be explained by the choice of probe and primers [5]. Indeed, the variable regions of the 16S rRNA gene that allow identification of bacteria have evolved at different evolutionary rates, leading to significant differences in base heterogeneity and phylogenetic discriminatory power [6]. Knowing that Clostridia belong to a complex phylogenetically heterogeneous group, with some clusters having high genetic strain diversity, we focused on *Clostridium sensu stricto* cluster I, in which *C. butyricum* is the type species [7]. We aligned the V3-V4 region of the *Clostridium* species in order to build the corresponding phylogenetic tree and compared it with that obtained from the V6 region sequences. We highlighted the *Clostridium* species

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**Figure 1.** Phylogenetic trees built from the alignment of sequences corresponding to the *Clostridium* V3-V4 and the V6 regions of the 16S rRNA gene. The V3-V4 (A) and V6 (B) regions of the 16S rRNA gene were selected from all *Clostridium* species (available in the Ribosomal Database Project [8]) by using the primers 341F-806R and 917F-1391R, respectively. We then used Molecular Evolutionary Genetics Analysis software to build the phylogenetic tree from the aligned sequences. We highlighted the *Clostridium* species belonging to cluster I, including *Clostridium perfringens* (orange), *Clostridium neonatale* (purple), and *Clostridium butyricum* (turquoise).
belonging to cluster I, including those previously associated with NEC in preterm neonates (ie, *C. butyricum*, *C. neonatale*, and *C. perfringens*) and noted that the phylogenetic distance between their V3-V4 regions was closer and consequently less discriminant when compared with their V6 regions [Figure 1]. Similarly, Chakravorty et al showed that the hypervariable region V6 (986–1043) was the shortest hypervariable region with the maximum degree of sequence heterogeneity within the genus *Clostridium* [9].

Second, most automated taxonomic assignment software, such as the Ribosomal Database Project (RDP), allows only limited identification from phylum to genus [10]. We matched the V3-V4 region of *C. butyricum* against the RDP database and obtained as first identification *Clostridium* sp., whereas *C. perfringens* was correctly identified. The V6 region allowed an accurate identification at the species level for both species.

In conclusion, we suggest that further studies exploring the gut microbiota associated with NEC include complementary culture-based methods to confirm the identification of bacteria at the species level.

**Note**

*Potential conflicts of interest.* All authors: No reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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