Molecular epidemiology of *Clostridium neonatale* and its relationship with the occurrence of necrotizing enterocolitis in preterm neonates

M. Hosny, E. Baptiste, A. Levasseur and B. La Scola

Aix-Marseille Université UM63, Institut de Recherche pour le Développement IRD 198, Assistance Publique, Hôpitaux de Marseille (AP-HM), Microbes, Evolution, Phylogeny and Infection (MEΦI), Institut Hospitalo-Universitaire (IHU), Méditerranée Infection, France

Abstract

Clostridia—especially *Clostridium butyricum*—are among the taxa most frequently identified from stool samples of preterm neonates with necrotizing enterocolitis (NEC). Recently, *Clostridium neonatale* has also been detected from epidemic cases, but using a culture-based approach we were unable to confirm this discovery in a local cohort. In order to investigate this link by a molecular approach, a specific *rpoB*-based quantitative real-time PCR was developed to detect *C. neonatale* directly from patients' stool specimens. Design of this *rpoB*-based quantitative real-time PCR was based on the genomic analysis of seven clinical isolates of *C. neonatale*. It was tested on stool samples from 88 preterm neonates with necrotizing enterocolitis and 71 matched controls. *C. neonatale* was significantly more prevalent in stools from preterm neonates with necrotizing enterocolitis than in controls (respectively 30/88 (34%) versus 9/71 (13%); p 0.003). Whole-genome analysis also allowed the identification of three genomic clusters of *C. neonatale*. This clustering was associated with a geographical location regardless of isolation from the NEC or control, suggesting asymptomatic carriage. Although less prevalent than *C. butyricum* in our cohort, *C. neonatale* is significantly associated with the occurrence of necrotizing enterocolitis.
© 2019 The Author(s). Published by Elsevier Ltd.

Corresponding author: B. La Scola, Pôle des Maladies Infectieuses, Aix-Marseille Université, IRD, Assistance Publique, Hôpitaux de Marseille (AP-HM), Microbes, Evolution, Phylogeny and Infection (MEΦI), Institut Hospitalo-Universitaire (IHU), Méditerranée Infection, 19-21 Boulevard Jean Moulin, 13385 Marseille Cedex 05, France.
E-mail: bernard.la-scola@univ-amu.fr

Introduction

Necrotizing enterocolitis (NEC) is a major cause of morbidity and mortality in neonatal intensive care units (NICUs), especially for very-low-birth-weight infants [1]. Over the past decade, most studies have focused on intestinal immaturity, feeding strategies, and the composition of the gut microbiota, as NEC is recognized as a multifactorial disease [1,2]. Approaches to determining the composition of the gut microbiota were performed using molecular tools such as 16S rRNA pyrosequencing, shotgun metagenomic sequencing, and specific quantitative real-time PCR (qPCR) [1,3,4]. These tools have been used to describe dysbiosis-linked alterations in neonates' gut microbiota. In addition, several bacterial species have been specifically associated with NEC; these include *Klebsiella pneumoniae*, *Enterobacter cloacae*, uropathogenic *Escherichia coli* (UPEC), *Clostridium butyricum* and *Clostridium neonatale* [1,4–7]. This latter was first isolated in the context of epidemic NEC cases in a Canadian NICU [8]. Pulsed-field gel electrophoresis identified a similar banding profile from blood and stool culture isolates [8]. Later, Roze et al. reported correlations between nutritional strategies and gut microbiota composition, where *C. neonatale* was identified from the first stool of preterm neonates with NEC [9], and its genome sequence was determined [10,11]. A causal relationship between the presence of *C. neonatale* in stool samples and NEC has not yet been clearly demonstrated, but it warrants further study. Based on a cohort

of neonates and controls enrolled to study the involvement of *C. butyricum* in NEC [3,7], we evaluated the association of *C. neonatale* with the occurrence of NEC. This hypothesis was developed by studying the frequency of *C. neonatale* in the neonates' stools using a specific *rpoB*-based qPCR, and then comparing the strains isolated by genomic sequencing.

Methods

Study design and patients

The study was validated by agreements from the ethics committee of the Institut Fédératif de Recherche, IFR48, and the Institut Hospitalo-Universitaire, IHU-2017-007. Written approval was obtained from the parents of all patients [3]. One hundred and fifty-nine stool samples were collected from preterm neonates whose parents had consented to the study; the neonates were treated in five NICUs. Enrolled samples were obtained from our previous cohort studies and included 88 patients with NEC and 71 healthy controls [3,7]. None of the patients were subjected to probiotic therapy. Stool samples were collected, if possible, on the day of symptom onset and stored at −80°C. Patients and controls were grouped by sex, gestational age (under 37 gestational weeks), birth weight, days of life, feeding strategies, mode of delivery, and previous antibiotic therapy (Table 1). Routine microbiological analysis was negative for all samples.

Genome sequencing, assembly and annotation of *Clostridium neonatale* isolates

Five *C. neonatale* strains—all from Marseille and isolated in a previous work—were sequenced and analysed [7]. Four were NEC-associated isolates (NEC25, NEC26, NEC32, NEC86) and one was from a control neonate (C25). Strains were cultured on 5% Columbia sheep-blood agar (Becton Dickinson®, USA) at 37°C for 48 h under anaerobic conditions before DNA extraction using an EZ1 DNA Tissue Kit (QIAGEN, Germany).

Genomic DNA was sequenced with MiSeq Technology (Illumina Inc., San Diego, CA, USA) using the paired-end applications. Strain NEC86 was additionally sequenced by MinION technology (Nanopore, Oxford, UK) [12,13]. SPAdes software was used for NEC86 reads assembly obtained from both sequencing methods [14]. Reads of the other *C. neonatale* genomes were mapped against the NEC86 dataset using the CLC genomics workbench 7 (Qiagen Inc., Valencia, CA, USA). Coding DNA sequences (CDSs) and annotation were procured using Prokka software [15]. Finally, sequenced genomes were deposited in the European Molecular Biology Laboratory, European Bioinformatics Institute (EMBL-EBI), under accession numbers: NEC25 (UWJD01000001−01000003), NEC26 (UICU01000001−01000003), NEC32 (UICW01000001−01000003), NEC86 (UICR01000001−01000003), and C25 (UICQ01000001−01000003) (Table 2). Further analysis and comparison were conducted by introducing genome sequences from two strains isolated from a Canadian NICU (LCDC99A005: GCA_001458595.1 and LCDC99A006: GCA_002553455.1) [8,11].

Design of specific qPCR for *Clostridium neonatale* detection

As previously performed for the detection of *C. butyricum* [3], we designed a specific qPCR targeting the *rpoB* gene of *C. neonatale*. First, we verified that a unique copy of the *rpoB* gene was present in the *C. neonatale* genome (see above). Second, these sequences were aligned with those of four pathogenic species using MEGA7 software [16]. Other *Clostridium* genomes included in this analysis were *C. butyricum* E4 str. BoNT E BL5262 (GCA_000182605.1), *Clostridium botulinum* A str. (GCA_000017025.1), *Clostridium perfringens* ATCC 13124 (GCA_000013285.1) and *Clostridioides (Clostridium) difficile* QCD-66c26 (GCA_000003215.1) as an outgroup at the class level (Clostridia). Primers and probe were designed to detect specifically the *C. neonatale rpoB* gene using Primer3 software [17]. Finally, a 151-nucleotide *rpoB* sequence was

TABLE 1. Factors associated with the occurrence of necrotizing enterocolitis (NEC) compared with control preterm neonates

Factors	NEC (n = 88) n (%)	Controls (n = 71) n (%)	Univariate analysis p value	Multivariate analysis OR (95%CI)	p value
Gestational age ± SD (days)	28.36 (±2.82)	28.24 (±2.93)	0.7864	NA	NA
Days of life ± SD (days)	25.99 (±13.16)	23.99 (±11.61)	0.3166	NA	NA
Male sex	49 (55.7)	43 (60.5)	0.594	NA	NA
Birth weight mean ± SD (g)	1179 (±380)	1174 (±432)	0.950	NA	NA
Very low birth weight (≤1500g)	69 (78.4)	58 (81.7)	0.617	NA	NA
Pasteurized breast milk	59 (67)	49 (69)	0.372	NA	NA
Formula fed	9 (10)	13 (18)	0.139	NA	NA
Vaginal delivery	32 (36)	37 (52)	0.023	2.3 [1.2-4.5]	0.018
Antibiotics before collection	64 (73)	40 (56)	0.092	1.9 [0.9-3.8]	0.07
Clostridium neonatale q-PCR-positive	30 (34)	9 (13)	0.003	3.7 [1.6-8.8]	0.003

SD, standard deviation; NA, not applicable.

TABLE 2. Characteristics of Clostridium neonatale draft genomes

Isolate	Location, date of isolation	Sequencing technique	Scaffold number	Genome length (base pair)	GC content (%)	ORFs number	Accession number
LCDC 99A005	NICU-1, 2000	Mate-pair	3	4 304 459	28.6	3795	GCA_001458595.1
LCDC 99A006	NICU-1, 2000	NA	58	4 658 596	28.4	4139	GCA_002553455.1
NEC25	NICU-3, December 2011	Paired-end	3	4 352 344	28.7	3883	UWJD01000001−01000003
NEC26	NICU-3, July 2012	Paired-end	3	4 282 644	28.9	3840	UICU01000001−01000003
NEC32	NICU-3, December 2012	Paired-end	3	4 321 416	28.7	3859	UICW01000001−01000003
NEC86	NICU-2, December 2010	Paired-end, Nanopore	3	4 739 641	28.6	4188	UICR01000001−01000003
C25	NICU-3, February 2012	Paired-end	3	4 358 232	28.6	3913	UICQ01000001−01000003

ORF, open-reading frame number; NEC, necrotizing enterocolitis; C25, control; NICU, neonatal intensive care unit; NICU-1, Canada; NICU-2, Marseille, France; NICU-3, Marseille, France; NA: not available.

selected; this region is homogenous among *C. neonatale* strains and highly heterogenic compared to that in other clostridia. This molecular construction was validated by a maximum-likelihood phylogenetic analysis generated by MEGA7 software (Fig. 1) [16]. LightCycler® 480 Probes Master (Roche, Germany) was used to achieve qPCR reactions. The optimized 20 μL qPCR mix contained: master mix (10 μL), primers (0.5 μL, 20 nM), probe (0.5 μL, 5 nM), water (3.5 μL) and DNA (5 μL). The qPCR cycling protocol started with one cycle of 50°C for 2 minutes, followed by one activation cycle (95°C for 5 min) and 40 amplification cycles (95°C for 1 sec; Tm 60°C for 30 sec). Tubes were placed in a thermal cycler CFX96 Touch™ (Bio-rad®, France). Sequences of the qPCR system were designed as follows: forward primer (AATAGTTGATAAA-GAACACGGTAGAGC), reverse primer (TAGCGGTTCT TTTGCTTGAG), and probe (FAM-TGCAGATGAAGAA-GATCAGTTTTT-TAMRA) (supplementary material Fig. S1). First, the qPCR system was tested on a collection of 79
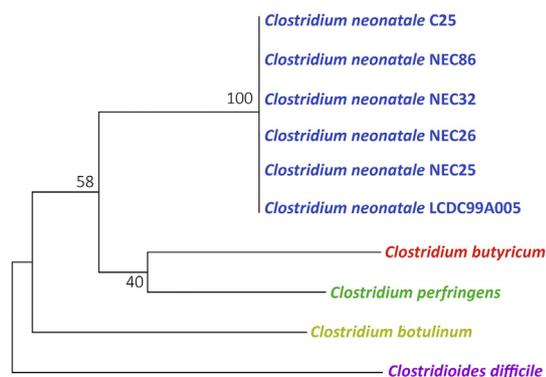
bacterial species associated with the gut microbiota, including five locally isolated *C. neonatale* strains and 24 *Clostridium* species (supplementary material Table S1). Total genomic DNA was extracted directly from stool samples using the NucleoSpin Tissue Kit (Macherey-Nagel, Hoerdt, France) as previously described [7].

Statistical analysis

Statistical analysis was performed using SPSS® statistics 2016 (IBM, NY, USA). Mean and standard deviation were used to describe continuous variables. Percentage and number of events were used for quantitative variables. The Student t-test or Mann−Whitney U test were used to perform two-group comparisons for quantitative variables. The χ-square (Mantel−Haenszel) test was used to perform two-group comparisons for qualitative variables, or the Fisher exact test was used when the expected count was <5. A multivariate analysis using logistic regression was performed to identify independent risk factors for NEC. Variables clinically relevant and associated ($p < 0.10$) with NEC in univariate analysis were used in the model. A p value < 0.05 was considered statistically significant.

FIG. 1. *In silico* analysis of *Clostridium neonatale* *rpoB*-specific region. This analysis was performed on the selected region of 151 nucleotides. Phylogenetic analysis showed that the *rpoB* sequence is homogenous among *C. neonatale* strains and classified it among *Clostridium* species. *C. difficile* was out-grouped. NEC, necrotizing enterocolitis; C25, control.

Draft genome-based and core-genome phylogeny

For draft genome-based phylogeny, only mapped genomes were included. Scaffolds were concatenated, and aligned using the MAFFT software [18]. For core genome analysis, a dataset comprising seven genomes was generated. Orthologous proteins were obtained from ProteinOrtho software using the following parameters: identity 50%, coverage 60% and E-value 1.10^{-10}. CDSs of core genes were inferred from the pangenome, then concatenated and aligned using a Python script. The phylogenetic trees were generated using the maximum-likelihood method within PhyML [19] and edited by TreeGraph 2 software [20]. BLAST was used to identify haemolysin (A, B, C), β-haemolysin sequences, *dlt* operon (*dlt* A, B, C, D) and the clusters of orthologous groups (COG) identification (E-value $1e^{-03}$, coverage 0,7 and identity percentage 30%).

Results

Evaluation of patient features and *Clostridium neonatale*-specific *rpoB*-based qPCR system

There was no statistically significant difference between NEC and control groups regarding gestational age, days of life, sex ratio, birth weight and feeding strategies. Only vaginal delivery was significantly more prevalent in controls than in NEC: 37/71 (52%) versus 32/88 (36%), $p < 0.05$. This statistical analysis improved the rationality of clinical features between NEC and healthy controls. Specificity of primers and probe were validated due to positive amplification for all *C. neonatale* strains (5/5) and the absence of amplification for other tested bacterial strains isolated from gut microbiota (0/74). This result excluded cross-reaction between the *C. neonatale* qPCR system and bacterial species inhabiting the gut microbiota. *C. neonatale* was more frequently detected in stools from preterm neonates with NEC than in controls: respectively 30/88 (34%) versus 9/71 (13%), p 0.003. The presence of *C. neonatale* in stool samples was significantly associated with the occurrence of NEC. Statistical results are summarized in Table 1. Means of *C. neonatale* qPCR cycle thresholds (Ct) were statistically non-significant between NEC and controls (26.9 and 25.4 respectively, p 0.5292, unpaired student *t* test), suggesting that the density of *C. neonatale* is similar in both cohorts. The prevalence of *C. neonatale* was also compared with that of *C. butyricum* reported in Hosny et al. [7]. In brief, the prevalence of *C. butyricum*, evaluated by both culture and qPCR, was assessed from each cohort and compared with the frequency of detection of *C. neonatale* (present work). In the majority of NEC cases, *C. neonatale* was frequently detected in association with *C. butyricum* when compared to controls: respectively 20/30, (66.7%) versus 1/9 (11.1%), p 0.003 (supplementary material Table S2).

Genomic analysis of *Clostridium neonatale*

Final draft genomes of *C. neonatale* isolates sequenced herein consist of three scaffolds. The average length of included genomes was 4 431 047 bp; NEC86 had the longest genome (4 739 641 bp) and NEC26 the shortest (4 282 644 bp). GC content varied between 28.4% (LCDC99A006) and 28.9% (NEC26). Predicted CDSs ranged between 3795 genes (LCDC99A005) and 4188 genes (NEC86) with an average of 3945. General features of these genomes are summarized in Table 2. The draft genome-based phylogeny of *C. neonatale* isolates allowed the identification of three clusters, revealing clonality among isolates from the same NICU by the following: (a) a strain isolated from NICU-1 (LCDC99A005), (b) a strain isolated from NICU-2 (NEC86) and (c) strains isolated from NICU-3 (NEC25, NEC26, NEC32 and C25) (Fig. 2A). Furthermore, core genome analysis distinguished the same clustering where the strict pangenome consisted of 5150 genes, including 3157 core genes (Fig. 2B). Predicted COG categories were homogenous in all *C. neonatale* genomes. The COGs encoding carbohydrate metabolism and transport, and therefore general functional prediction (G and R), were overrepresented; in contrast, no treatment or modification of RNA, and therefore the structure and dynamics of chromatin, were identified (Fig. 2A and B) (supplementary material Fig. S2 and Table S3).

Identification of virulent and unique genes

Comparative analyses identified haemolysin-encoding genes in all strains as follows: haemolysin A (coverage: 100%, identity: 84−92%), two protein sequences encoded for haemolysin B (coverage: 100%, identity: 44−99%) for the first protein and (coverage: 99%, identity: 76−77%) for the second one, haemolysin C (coverage: 96%, identity: 81%) and β-haemolysin (coverage: 100%, identity: 92%). We also identified genes
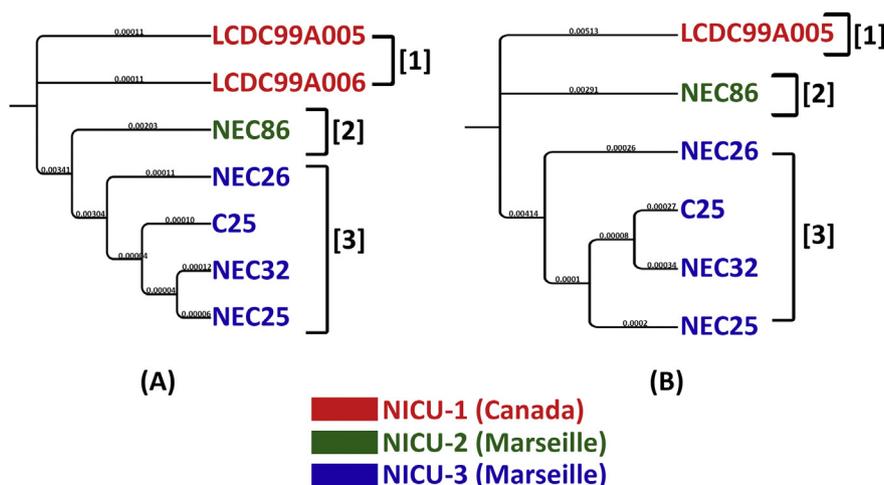

FIG. 2. Relationship of Clostridium neonatale strains based on genomic analysis. (A) Phylogenetic tree based on core genome. (B) Phylogenetic tree based on whole-genome alignment. NEC, necrotizing enterocolitis; C25, control.

coding for the secretion of *C. difficile* toxins A and B. Moreover, sequences of the *dlt* operon (*dlt A, B, C, D*) were not detected in all draft genomes of *C. neonatale* (supplementary material Table S4). Unique genes also existed and mostly represented hypothetical proteins, as follows: NEC25 (1/4, 25%), NEC26 (77/113, 68%), NEC32 (28/39, 72%), C25 (2/3, 67%), NEC86 (139/212, 65.5%) and LCDC99A005 (31/59, 52.5%) LCDC99A006 (supplementary material Table S5).

Discussion

Dysbiosis remains a major risk factor inducing the establishment of NEC by promoting the translocation of pathogenic bacteria. Specific microorganisms were involved: the predominance of γ-proteobacteria, generating an excessive inflammatory response, and irregular colonization by strictly anaerobic bacteria, including clostridia [1]. Multidisciplinary approaches have indicated a link between this class—especially *C. butyricum*, *C. neonatale* and *C. perfringens*—and the occurrence of NEC [1,21]. To date, *C. butyricum* is the only *Clostridium* species among the cases of clostridia-associated NEC which has been clearly described as correlating with NEC [3,7,22]. However, several studies have reported the involvement of *C. neonatale* in NEC [1,8]. Using a culture-based strategy, we observed that the prevalence of *C. neonatale* in stool samples from patients with NEC was not significantly different from that of controls: respectively 4/88 (4.5%) versus 1/71 (1.14%), p 0.26 [7]. In a review of the previous work it was proposed that this discrepancy could be due to the use of a heat-shock-based protocol. If this is supposed to kill all bacteria except spore-formers such as *C. neonatale,* we could not exclude that the method avoids the isolation of *in vivo* non-spore-forming *Clostridium* species. Therefore, we decided to investigate our cohort using *C. neonatale*-specific *rpoB*-based qPCR. With a strong phylogeny and taxonomy index, *rpoB* is a conserved gene whose efficacy for the identification of fastidious microorganisms directly from samples has been previously demonstrated [23,24] and which was used for the heterogeneous detection of BoNT-producing clostridia [25] and *C. butyricum* directly from stool samples [3]. A significant frequency of *C. neonatale* in stools from NEC was detected compared to controls (respectively 34% versus 13%, p 0.003). In contrast with the study conducted by Rozé et al., in which the authors reported links between feeding strategies and NEC and the abundance of *C. neonatale* in NEC patients [9], the feeding strategy was herein not associated with the development of NEC. The only correlation identified was vaginal delivery, a feature already observed in NEC cases [2]. Beside *C. neonatale* and *C. butyricum*, several NEC outbreaks have been reported to be associated

with other bacterial species [1,2]. A case report described toxin-producing *C. perfringens* where NEC severity was independent of the α-toxin concentration [26]. Also, nosocomial colonization by *Klebsiella pneumoniae* type 26 has been reported and antibiotic typing was used to specify strain characteristics [27]. Furthermore, genetically similar clones of *Enterobacter sakazakii* were distinguished from powdered milk formula and neonates with NEC [28]. A few other bacterial species—such as UPEC and *C. paraputrificum*—have been suspected of being associated with NEC [4,29].

The use of whole-genome sequencing allowed us to further compare and characterize isolates even if the number of strains and available genomes was limited. This study disclosed genetic similarity between strains isolated from the same NICU. Through phylogenetic analysis, we identified three distinct clusters, highlighted by their geographic areas of isolation, suggesting the same clone of *C. neonatale* spreading in the same NICU-3 as we previously observed with *C. butyricum* [7]. This is in agreement with the discovery of this species, as it was first isolated from a Canadian NICU (strains LCDC), and clonality between isolates was proved by pulsed-field gel electrophoresis from stool and blood cultures [8]. Geographic and temporal clustering of NECs were described in several studies with various aetiologies. Temporal clustering was reported by Faustini et al. in diverse NICUs [30]. The similarity between control (C25) and NEC-associated *C. neonatale* in cluster [3] presupposes the existence of asymptomatic carriage. This same kind of mechanism was described in the case of *C. difficile*-associated pseudomembranous colitis [31], and we suggested a similar mechanism for *C. butyricum* in neonates [7]. It should be noted that there appears to be a strong association between *C. neonatale* and neonates/children as it is a species that has never been isolated from adults in our microbiota studies [32]. On the contrary, *C. neonatale* has been identified by sequencing of 16S rRNA in the microbiota of young children and has a strong association with developing asthma [33].

If the association between clostridia and NEC is increasingly being reported, the pathogeny of this disease remains elusive in spite of the usual suggestion of toxin production, as in the case of *C. difficile* infections (CDIs) [33]. Herein, genes encoding the secretion of bacterial toxins were predicted, especially haemolysin and *C. difficile* toxins A/B (TcdA/B). These latter are a leading cause of CDI, where the pathogenic mechanism is the consequence of TcdA (enterotoxin) and TcdB (cytotoxin) production inducing colonic tissue damage [25,26]. The idea of toxin-mediated disease rather than invasion-mediated disease is supported by the work of Heida et al. [34]. Furthermore, haemolysin sequences with a highly conserved domain found in a toxin of *Brachyspira hyodysenteriae* showed a cytolytic effect in

several cell lines [13]. Cassir et al. identified cytotoxic activity of *C. butyricum* supernatant on Jurkat cells [4].

In conclusion, this study highlights the association between *C. neonatale* and NEC and the possible existence of NEC-associated geographic clones. Further genomic analysis is required on a larger number of sequenced genomes.

Conflict of interest

Acknowledgements

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nmni.2019.100612.

References

[1] Hosny M, Cassir N, La Scola B. Updating on gut microbiota and its relationship with the occurrence of necrotizing enterocolitis. Hum Microbiome J 2017;4:14–9. https://doi.org/10.1016/j.humic.2017.04.001.

[2] Cassir N, Simeoni U, La Scola B. Gut microbiota and the pathogenesis of necrotizing enterocolitis in preterm neonates. Future Microbiol 2016;11:273–92. https://doi.org/10.2217/fmb.15.136.

[3] Cassir N, Benamar S, Khalil JB, Croce O, Saint-Faust M, Jacquot A, et al. *Clostridium butyricum* strains and dysbiosis linked to necrotizing enterocolitis in preterm neonates. Clin Infect Dis 2015;61:1107–15. https://doi.org/10.1093/cid/civ468.

[4] Ward DV, Scholz M, Zolfo M, Taft DH, Schibler KR, Tett A, et al. Metagenomic sequencing with strain-level resolution implicates uropathogenic *E. coli* in necrotizing enterocolitis and mortality in preterm infants. Cell Rep 2016;14:2912–24.

[5] Boccia D, Stolfi I, Lana S, Moro ML. Nosocomial necrotising enterocolitis outbreaks:epidemiology and control measures. Eur J Pediatr 2001;160:385–91.

[6] Smith B, Bodé S, Petersen BL, Jensen TK, Pipper C, Kloppenborg J, et al. Community analysis of bacteria colonizing intestinal tissue of neonates with necrotizing enterocolitis. BMC Microbiol 2011;11. https://doi.org/10.1186/1471-2180-11-73.

[7] Hosny M, Bou Khalil JY, Caputo A, Abdallah RA, Levasseur A, Colson P, et al. Multidisciplinary evaluation of *Clostridium butyricum* clonality isolated from preterm neonates with necrotizing enterocolitis in South France between 2009 and 2017. Sci Rep 2019;9:2077. https://doi.org/10.1038/s41598-019-38773-7.

[8] Alfa MJ, Robson D, Davi M, Bernard K, van Caeseele P, Harding GKM. An outbreak of necrotizing enterocolitis associated with a novel *Clostridium* species in a neonatal intensive care unit. Clin Infect Dis 2002;35:101–5.

[9] Roze J-C, Ancel P-Y, Lepage P, Martin-Marchand L, Al Nabhani Z, Delannoy J, et al. Nutritional strategies and gut microbiota composition as risk factors for necrotizing enterocolitis in very-preterm infants. Am J Clin Nutr 2017;36:821–30. https://doi.org/10.3945/ajcn.

[10] Benamar S, Cassir N, La Scola B. Genome sequence of a *Clostridium neonatale* strain isolated in a Canadian neonatal intensive care unit. Genome Announc 2016;4. https://doi.org/10.1128/genomeA.01431-15.

[11] Bernard K, Burdz T, Wiebe D, Alfa M, Bernier A-M. *Clostridium neonatale* sp. nov. linked to necrotizing enterocolitis in neonates and a clarification of species assignable to the genus *Clostridium* (Prazmowski 1880) emend. Lawson and Rainey 2016. Int J Syst Evol Microbiol 2018;68:2416–23. https://doi.org/10.1099/ijsem.0.002827.

[12] Lu H, Giordano F, Ning Z. Oxford Nanopore MinION sequencing and genome assembly. Genomics, Proteomics Bioinforma 2016;14:265–79. https://doi.org/10.1016/j.gpb.2016.05.004.

[13] Hosny M, Benamar S, Durand G, Armstrong N, Michelle C, Cadoret F, et al. Description of *Clostridium phoceensis* sp. nov., a new species within the genus *Clostridium*. New Microbe. New Infect 2016;14:85–92. https://doi.org/10.1016/j.nmni.2016.09.008.

[14] Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, et al. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol 2012;19:455–77. https://doi.org/10.1089/cmb.2012.0021.

[15] Seemann T. Prokka: rapid prokaryotic genome annotation. Bioinformatics 2014;30:2068–9. https://doi.org/10.1093/bioinformatics/btu153.

[16] Kumar S, Stecher G, Tamura K. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol 2016;33:1870–4. https://doi.org/10.1093/molbev/msw054.

[17] Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, et al. Primer3—new capabilities and interfaces. Nucleic Acids Res 2012;40:1–12. https://doi.org/10.1093/nar/gks596.

[18] Katoh K, Standley DM. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol Biol Evol 2013;30:772–80. https://doi.org/10.1093/molbev/mst010.

[19] Guindon S, Gascuel O. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst Biol 2003;52:696–704. https://doi.org/10.1080/10635150390235520.

[20] Stöver BC, Müller KF. TreeGraph 2: combining and visualizing evidence from different phylogenetic analyses. BMC Bioinform 2010;11:7. https://doi.org/10.1186/1471-2105-11-7.

[21] Zhao-Fleming H, Dissanaike S, Rumbaugh K. Are anaerobes a major, underappreciated cause of necrotizing infections? Anaerobe 2017;45:65–70. https://doi.org/10.1016/j.anaerobe.2017.04.012.

[22] Benamar S, Cassir N, Merhej V, Jardot P, Robert C, Raoult D, et al. Multi-spacer typing as an effective method to distinguish the clonal lineage of *Clostridium butyricum* strains isolated from stool samples

during a series of necrotizing enterocolitis cases. J Hosp Infect 2017;95:300–5. https://doi.org/10.1016/j.jhin.2016.10.026.

[23] Adékambi T, Drancourt M, Raoult D. The rpoB gene as a tool for clinical microbiologists. Trends Microbiol 2009;17:37–45. https://doi.org/10.1016/j.tim.2008.09.008.

[24] Fenollar F, Raoult D. Molecular genetic methods for the diagnosis of fastidious microorganisms. APMIS 2004;112:785–807. https://doi.org/10.1111/j.1600-0463.2004.apm11211-1206.x.

[25] Fach P, Micheau P, Mazuet C, Perelle S, Popoff M. Development of real-time PCR tests for detecting botulinum neurotoxins A, B, E, F producing *Clostridium botulinum, Clostridium baratii* and *Clostridium butyricum*. J Appl Microbiol 2009;107:465–73. https://doi.org/10.1111/j.1365-2672.2009.04215.x.

[26] Dittmar E, Beyer P, Fischer D, Schäfer V, Schoepe H, Bauer K, et al. Necrotizing enterocolitis of the neonate with *Clostridium perfringens*: diagnosis, clinical course, and role of alpha toxin. Eur J Pediatr 2008;167:891–5. https://doi.org/10.1007/s00431-007-0614-9.

[27] Hill HR, Hunt CE, Matsen JM. Nosocomial colonization with *Klebsiella*, type 26, in a neonatal intensive-care unit associated with an outbreak of sepsis, meningitis, and necrotizing enterocolitis. J Pediatr 1974;85: 415–9. https://doi.org/10.1016/S0022-3476(74)80133-2.

[28] vanAcker J, de Smet F, Muyldermans G, Naessens A, Lauwers S, Muyldermans T, et al. Outbreak of necrotizing enterocolitis associated with *Enterobacter sakazakii* in powdered milk formula. J Clin Microbiol 2001;39:7–12. https://doi.org/10.1128/JCM.39.1.293.

[29] Ferraris L, Butel MJ, Campeotto F, Vodovar M, Rozé JC, Aires J. Clostridia in premature neonates' gut: incidence, antibiotic susceptibility, and perinatal determinants influencing colonization. PLoS One 2012;7. https://doi.org/10.1371/journal.pone.0030594.

[30] Faustini A, Forastiere F, Giorgi Rossi P, Perucci CA. An epidemic of gastroenteritis and mild necrotizing enterocolitis in two neonatal units of a University Hospital in Rome, Italy. Epidemiol Infect 2004;132: 455–65.

[31] Eyre DW, Cule ML, Wilson DJ, Griffiths D, Vaughan A, O'Connor L, et al. Diverse sources of *C. difficile* infection identified on whole-genome sequencing. N Engl J Med 2013;369:1195–205. https://doi.org/10.1056/NEJMoa1216064.

[32] Lagier JC, Dubourg G, Million M, Cadoret F, Bilen M, Fenollar F, et al. Culturing the human microbiota and culturomics. Nat Rev Microbiol 2018;16:540–50. https://doi.org/10.1038/s41579-018-0041-0.

[33] Stiemsma LT, Arrieta M-C, Dimitriu PA, Cheng J, Thorson L, Lefebvre DL, et al. Shifts in *Lachnospira* and *Clostridium* sp. in the 3-month stool microbiome are associated with preschool age asthma. Clin Sci 2016;130:2199–207. https://doi.org/10.1042/CS20160349.

[34] Heida FH, Harmsen HJM, Timmer A, Kooi EMW, Bos AF, Hulscher JBF. Identification of bacterial invasion in necrotizing enterocolitis specimens using fluorescent in situ hybridization. J Perinatol 2017:1–6. https://doi.org/10.1038/jp.2016.165.