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

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**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.

**Keywords:** Clonal lineage, *Clostridium neonatale*, Core-genome phylogeny, Necrotizing enterocolitis, rpoB-based specific quantitative real-time polymerase chain reaction

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**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 analyzed [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 strain, BoNT E BL5262 (GCA_000182605.1), *Clostridium botulinum* A strain (GCA_000017025.1), *Clostridium perfringens* ATCC 13124 (GCA_000013285.1) and *Clostridoides (Clostridium)* difficile QCD-6626 (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) | Controls (n = 71) | Univariate analysis | Multivariate analysis |
|----------------------------------------------|--------------|-------------------|---------------------|----------------------|
| Gestational age ± SD (days)                  | 28.36 ±2.82  | 28.24 ±2.93       | 0.7864              | NA                   |
| Days of life ± SD (days)                     | 25.99 ±13.16 | 23.99 ±11.61      | 0.3166              | NA                   |
| Male sex                                     | 49 (55.7)    | 43 (60.5)         | 0.594               | NA                   |
| Birth weight mean ± SD (g)                  | 1179 (±380)  | 1174 (±432)       | 0.950               | NA                   |
| Very low birth weight (<1500g)              | 69 (78.4)    | 58 (81.7)         | 0.617               | NA                   |
| Pasteurized breast milk                      | 59 (67)      | 49 (69)           | 0.372               | NA                   |
| Formula fed                                  | 9 (10)       | 13 (18)           | 0.139               | 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_00253455.1   |
| NEC25         | NICU-3, December 2011       | Paired-end           | 3              | 4 352 344                 | 28.7          | 3883        | UWVJO01000001−10000003 |
| NEC26         | NICU-3, July 2012           | Paired-end           | 3              | 4 282 644                 | 28.9          | 3840        | UICU01000001−10000003 |
| NEC27         | NICU-3, December 2012       | Paired-end           | 3              | 4 321 416                 | 28.7          | 3859        | LCICW01000001−10000003 |
| NEC86         | NICU-2, December 2010       | Paired-end, Nanopore | 3              | 4 739 641                 | 28.6          | 4188        | UICR01000001−10000003 |
| C25           | NICU-3, February 2012       | Paired-end           | 3              | 4 358 232                 | 28.6          | 3913        | UICQ01000001−10000003 |

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 [16]. LightCycler® 480 Probes Master (Roche, Germany) was used to achieve qPCR reactions. The optimized 20 μL qPCR mix contained: master mix (10 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-GAACACGGTAGAG), reverse primer (TAGCGGTTCTGAACACGGTAGAG), and probe (FAM-TGCAGATGAAGAAACACGGTAGAGC) (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.

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 pan-genome, then concatenated and aligned using a Python script. The phylogenetic trees were generated using the maximum-likelihood method within PhyML [19] and edited by TreelGraph 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%).

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.
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 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 over-represented; 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...
coding for the secretion of \textit{C. difficile} toxins A and B. Moreover, sequences of the \textit{dlt} operon (\textit{dlt} A, B, C, D) were not detected in all draft genomes of \textit{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, 65.8%) 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 \textit{γ}-proteobacteria, generating an excessive inflammatory response, and irregular colonization by strictly anaerobic bacteria, including \textit{clostridia} \cite{1}. Multidisciplinary approaches have indicated a link between this class—especially \textit{C. butyricum}, \textit{C. neonatale} and \textit{C. perfringens}—and the occurrence of NEC \cite{1,121}. To date, \textit{C. butyricum} is the only \textit{Clostridium} species among the cases of \textit{clostridia}-associated NEC which has been clearly described as correlating with NEC \cite{3,7,22}. However, several studies have reported the involvement of \textit{C. neonatale} in NEC \cite{1,8}. Using a culture-based strategy, we observed that the prevalence of \textit{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%), \(p = 0.26\) \cite{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 \textit{C. neonatale}, we could not exclude that the method avoids the isolation of \textit{in vivo} non-spore-forming \textit{Clostridium} species. Therefore, we decided to investigate our cohort using \textit{C. neonatale}-specific \textit{rpoB}-based qPCR. With a strong phylogeny and taxonomy index, \textit{rpoB} is a conserved gene whose efficacy for the identification of fastidious microorganisms directly from samples has been previously demonstrated \cite{23,24} and which was used for the heterogeneous detection of BoNT-producing \textit{clostridia} \cite{25} and \textit{C. butyricum} directly from stool samples \cite{3}. A significant frequency of \textit{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 \textit{C. neonatale} in NEC patients \cite{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 \cite{2}. Beside \textit{C. neonatale} and \textit{C. butyricum}, several NEC outbreaks have been reported to be associated with other bacterial species \cite{1,2}. A case report described toxin-producing \textit{C. perfringens} where NEC severity was independent of the \(α\)-toxin concentration \cite{26}. Also, nosocomial colonization by \textit{Klebsiella pneumoniae} type 26 has been reported and antibiotic typing was used to specify strain characteristics \cite{27}. Furthermore, genetically similar clones of \textit{Enterobacter sakazakii} were distinguished from powdered milk formula and neonates with NEC \cite{28}. A few other bacterial species—such as \textit{UPEC} and \textit{C. paraputrificum}—have been suspected of being associated with NEC \cite{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 \textit{C. neonatale} spreading in the same NICU-3 as we previously observed with \textit{C. butyricum} \cite{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 \cite{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 \cite{30}. The similarity between control (C25) and NEC-associated \textit{C. neonatale} in cluster \cite{3} presupposes the existence of asymptomatic carriage. This same kind of mechanism was described in the case of \textit{C. difficile}-associated pseudomembranous colitis \cite{31}, and we suggested a similar mechanism for \textit{C. butyricum} in neonates \cite{7}. It should be noted that there appears to be a strong association between \textit{C. neonatale} and neonates/children as it is a species that has never been isolated from adults in our microbiota studies \cite{32}. On the contrary, \textit{C. neonatale} has been identified by sequencing of 16S rRNA in the microbiota of young children and has a strong association with developing asthma \cite{33}.

If the association between \textit{clostridia} and NEC is increasingly being reported, the pathogenicity of this disease remains elusive in spite of the usual suggestion of toxin production, as in the case of \textit{C. difficile} infections (CDIs) \cite{33}. Herein, genes encoding the secretion of bacterial toxins were predicted, especially haemolysin and \textit{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 \cite{25,26}. The idea of toxin-mediated disease rather than invasion-mediated disease is supported by the work of Heida et al. \cite{34}. Furthermore, haemolysin sequences with a highly conserved domain found in a toxin of \textit{Brachyspira hydysenteriae} 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**

None of the authors have any conflicts of interest to report. This work was supported by the French Government under the Investissements d’Avenir programme managed by the Agence Nationale de la Recherche (ANR) (reference: Méditerranée-Infection 10-IAHU-03), by Région Provence-Alpes-Côte d’Azur and European funding FEDER PRIMI. M. Hosny was supported by Fondation de Coopération Scientifique Méditerranée-Infection (Infectiopôle-Sud 2015).

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**Appendix A. Supplementary data**

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

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