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1 **Association between *Clostridium neonatale* and the occurrence of Necrotizing Enterocolitis**  
2 **in preterm neonates**

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14 **Running title:**

15 **Keywords:** Necrotizing enterocolitis; *Clostridium neonatale*; rpoB-based specific quantitative  
16 real-time polymerase chain reaction; core-genome phylogeny; clonal lineage; whole genome  
17 sequence

## 1 **Abstract**

2 Clostridia are among taxa most frequently identified from necrotizing enterocolitis preterm  
3 neonates' stool samples, especially *Clostridium butyricum*. Recently, *Clostridium neonatale* has  
4 also been detected from epidemic cases, but using a culture-based approach, we were unable to  
5 confirm this discovery in a local cohort. In order to investigate this link by a molecular approach,  
6 a specific *rpoB*-based quantitative real-time polymerase chain reaction was developed to detect  
7 *C. neonatale* directly from patients' stool specimens. Design of this *rpoB*-based quantitative real-  
8 time polymerase was based on the genomic analysis of seven clinical isolates of *C. neonatale*. It  
9 was tested on stool samples from 88 preterm neonates with necrotizing enterocolitis and 71  
10 matched controls. *C. neonatale* was significantly more prevalent in stools from preterm neonates  
11 with necrotizing enterocolitis than in controls (respectively 30/88 [34%]; vs. 9/71 [13%];  $p =$   
12 0.003). Whole-genome analysis also allowed the identification of three genomic clusters of *C.*  
13 *neonatale*. This clustering was associated with a geographical location regardless of isolation in  
14 the NEC or control, suggesting asymptomatic carriage. Although less prevalent than *C.*  
15 *butyricum* in our cohort, *C. neonatale* is significantly associated with the occurrence of  
16 necrotizing enterocolitis.

17 **Keywords:** Necrotizing enterocolitis, *Clostridium neonatale*, *rpoB*-based specific quantitative  
18 real-time polymerase chain reaction, core-genome phylogeny, clonal lineage.

## 19 **Introduction**

20 Necrotizing enterocolitis (NEC) is a major cause of morbidity and mortality in neonatal  
21 intensive care units (NICUs), especially for very low birth weight born (VLBW) [1]. Over the  
22 past decade, most studies have focused on intestinal immaturity, feeding strategies and the  
23 composition of the gut microbiota, as NEC is recognized as a multifactorial disease [1,2].  
24 Approaches of gut microbiota composition determination was performed using molecular-based  
25 tools, such as 16S rRNA pyrosequencing, shotgun metagenomic sequencing and specific  
26 quantitative real-time polymerase chain reaction [1,3,4]. These tools have been used to describe  
27 dysbiosis-linked alterations in neonates' gut microbiota. In addition, several bacterial species  
28 have been specifically associated with NEC including *K. pneumoniae*, *E. cloacae*,  
29 Uropathogenic *Escherichia coli*, *Clostridium butyricum* and *Clostridium neonatale* [1,4–7]. This  
30 latter was first isolated in the context of epidemic NEC cases in a Canadian NICU [8]. Pulsed-  
31 field gel electrophoresis identified similar banding profile from blood and stool culture isolates  
32 [8]. Later, Roze *et al.* reported correlations between nutritional strategies and gut microbiota  
33 composition, where *C. neonatale* was identified from first stool of preterm neonates with NEC  
34 [9] and its genome sequence was determined [10,11]. A causal relationship between the  
35 presence of *C. neonatale* in stool samples and NEC has not yet been clearly demonstrated, but it  
36 warrants further studies. Based on a cohort of neonates and controls enrolled to study the  
37 involvement of *C. butyricum* with NEC [3,7], we evaluated the association of *C. neonatale* in  
38 the occurrence of NEC. This hypothesis was developed by studying the frequency of *C.*  
39 *neonatale* in the neonates' stools using a specific *rpoB*-based quantitative real-time polymerase  
40 chain reaction (qPCR), and then comparing the strains isolated by genomic sequencing.

## 41 **Materials and Methods**

## 42 **Study design and patients**

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

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

54 Five *C. neonatale* strains, all from Marseille, isolated in a previous work were sequenced and  
55 analyzed [7]. Four were NEC-associated isolates (NEC25, NEC26, NEC32, NEC86) and one  
56 was from a control neonate (C25). Strains were cultured on 5% Columbia sheep blood agar  
57 (Becton Dickinson®, USA) at 37°C for 48 hours under anaerobic conditions before DNA  
58 extraction on EZ1 DNA Tissue Kit (QIAGEN, Germany). Genomic DNA was sequenced on  
59 MiSeq Technology (Illumina, Inc., San Diego, CA) using the paired-end applications. NEC86  
60 strain was additionally sequenced by MinION technology (Nanopore, Oxford, UK) [12,13].  
61 SPAdes software was used for NEC86 reads assembly obtained from both sequencing methods  
62 [14]. Reads of the other *C. neonatale* genomes, were mapped against NEC86 dataset using the  
63 CLC genomics workbench 7 (Qiagen Inc., Valencia, CA). Coding DNA sequences (CDS) and  
64 annotation were procured using Prokka software [15]. Finally, sequenced genomes were

65 deposited to the European Molecular Biology Laboratory – European Bioinformatics Institute  
66 (EMBL-EBI) under accession numbers: NEC25 (UWJD01000001- 01000003), NEC26  
67 (UICU01000001- 01000003), NEC32 (UICW01000001- 01000003), NEC86 (UICR01000001-  
68 01000003), C25 (UICQ01000001- 01000003) (Table 2). Further analysis and comparison was  
69 conducted by introducing genome sequences from two strains isolated from a Canadian NICU  
70 (LCDC99A005: GCA\_001458595.1 and LCDC99A006: GCA\_002553455.1) [8,11].

### 71 **Design of specific qPCR for *Clostridium neonatale* detection**

72 As previously performed for the detection of *C. butyricum* [3], we designed a *C. neonatale*  
73 specific qPCR targeting the *rpoB* gene. In a first time, we verified that the *rpoB* gene was  
74 presented in unique copy within *C. neonatale* genomes (see above). In a second time, these  
75 sequences were aligned with those of 4 pathogenic species using MEGA7 software [16]. Other  
76 *Clostridium* genomes included in this analysis were *C. butyricum* E4 str. BoNT E BL5262  
77 (GCA\_000182605.1), *Clostridium botulinum* A str. (GCA\_000017025.1), *Clostridium*  
78 *perfringens* ATCC 13124 (GCA\_000013285.1) and *Clostridioides (Clostridium) difficile* QCD-  
79 66c26 (GCA\_000003215.1) as an outgroup at the class level (Clostridia). Primers and probe  
80 were designed to detect specifically *C. neonatale rpoB* gene using Primer3 software [17].  
81 Finally, a 151 nucleotides *rpoB* sequence was selected: this region is homogenous among *C.*  
82 *neonatale* strains and highly heterogenic compared to other Clostridia. This molecular  
83 construction was validated by a Maximum-Likelihood phylogenetic analysis generated by  
84 MEGA7 software (Figure 1) [16]. LightCycler<sup>®</sup> 480 Probes Master (Roche, Germany) was used  
85 to achieve qPCR reactions. The optimized 20  $\mu$ L qPCR mix contained: master mix (10  $\mu$ L),  
86 primers (0.5  $\mu$ L, 20 nM), probe (0.5  $\mu$ L, 5 nM), water (3.5  $\mu$ L) and 5  $\mu$ L of DNA. The qPCR

87 cycling protocol started with one cycle of 50°C for 2 minutes, followed by one activation cycle  
88 (95°C for 5 min) and 40 amplification cycles (95°C for 1 second; T<sub>m</sub>= 60°C for 30 seconds).  
89 Tubes were deposited in a thermal cycler CFX96 Touch™ (Bio-rad®, France). Sequences of the  
90 qPCR system were designed as follows: forward primer:  
91 AATAGTTGATAAAGAACACGGTAGAGC, reverse primer: TAGCGGTTCTTTTGCTTGAG  
92 and probe (FAM-TGCAGATGAAGAAGATCAGTTTTT-TAMRA) (Supplementary Figure  
93 S1). First, the qPCR system was tested on a collection of 79 bacterial species associated with the  
94 gut microbiota, including five locally isolated *C. neonatale* and 24 *Clostridium* species  
95 (Supplementary Table S1). Total genomic DNA was extracted directly from stool samples using  
96 the NucleoSpin Tissue Kit (Macherey-Nagel, Hoerd, France) as previously described [7].

#### 97 **Statistical analysis**

98 Statistical analysis was performed using SPSS® statistics 2016 (IBM, NY, USA). Mean and  
99 standard deviation were used to describe continuous variables. Percentage and number of events  
100 were used for quantitative variables. Student t-test or Mann-Whitney *U* test were used to perform  
101 two-group comparisons for quantitative variables. The chi-square (Mantel-Haenszel) test was  
102 used to perform two-group comparisons for qualitative variables, or the Fisher exact test was  
103 used when the expected count was less than 5. A multivariate analysis using logistic regression  
104 was performed to identify independent risk factors for NEC. Variables clinically relevant and  
105 associated ( $p < 0.10$ ) with NEC in univariate analysis were used in the model. A  $p$  value of <  
106 0.05 was considered statistically significant.

#### 107 **Draft genome-based and core-genome phylogeny**

108 For draft genome-based phylogeny, only mapped genomes were included. Scaffolds were  
109 concatenated, and aligned using the MAFFT software [18]. For core-genome analysis, a dataset

110 comprising seven genomes were generated. Orthologous proteins were obtained from  
111 ProteinOrtho software using the following parameters: identity 50%, coverage 60% and E-value  
112  $1.10^{-10}$ . CDSs of core-genes were inferred from the pan-genome, then concatenated and aligned  
113 using a Python script. The phylogenetic trees were generated using the maximum-likelihood  
114 method within PhyML [19] and edited by TreeGraph 2 software [20]. BLAST was used to  
115 identify hemolysin (A, B, C), beta-hemolysin sequences, *dlt* operon (*dlt A, B, C, D*) and the  
116 Clusters of Orthologous Groups (COG) identification (E-value  $1e^{-03}$ , coverage 0,7 and identity  
117 percent 30% for this later).

## 118 **Results**

### 119 **Evaluation of patients features and *Clostridium neonatale* specific *rpoB*-based qPCR system**

120 There was no statistically significant difference between NEC and control groups regarding  
121 gestational age, days of life, sex ratio, birth weight and feeding strategies. Only vaginal delivery  
122 was significantly more prevalent in controls than in NEC (37/71 [52%]; vs 32/88 [36%];  $p <$   
123 0.05). This statistical analysis improved the rationality of clinical features between NEC and  
124 healthy controls. Specificity of primers and probe were validated due to positive amplification  
125 for all *C. neonatale* strains (5/5) and the absence of amplification for other bacterial strains tested  
126 isolated from gut microbiota (0/74). This result excluded cross-reaction between *C. neonatale*  
127 qPCR system and bacterial species inhabited within the gut microbiota. *C. neonatale* was more  
128 frequently detected in stools from preterm neonates with NEC than in controls (respectively  
129 30/88 [34%]; vs. 9/71 [13%];  $p = 0.003$ ). The presence of *C. neonatale* in stool samples was  
130 significantly associated with the occurrence of NEC. Statistical results are summarized in Table  
131 1. Means of *C. neonatale* qPCR cycle thresholds (Ct) were statistically non-significant between  
132 NEC and controls (26.9 and 25.4 respectively,  $p$  value = 0.5292, unpaired student *t* test),



133 suggesting that the density of *C. neonatale* is similar in both cohorts. The prevalence of *C.*  
134 *neonatale* was also evaluated with that of *C. butyricum* reported in Hosny *et al.* [7]. In brief, the  
135 prevalence of *C. butyricum*, evaluated by both culture and qPCR, were assessed from each  
136 cohort and compared with *C. neonatale* frequency of detection (present work). In the majority of  
137 NEC cases, *C. neonatale* was frequently detected in association with *C. butyricum* when  
138 compared to controls (respectively 20/30, [66.7%] vs 1/9, [11.1%],  $p=0.003$ ) (Supplementary  
139 Table S2).

#### 140 **Genomic analysis of *Clostridium neonatale***

141 Final draft genomes of *C. neonatale* isolates sequenced herein consist of 3 scaffolds. The  
142 average length of included genomes was 4,431,047 bp, NEC86 had the longest genome length  
143 (4,739,641 bp) and NEC26 the shortest one (4,282,644 bp). GC content varied between 28.4%  
144 (LCDC99A006) and 28.9% (NEC26). Predicted CDSs ranged between 3,795 genes  
145 (LCDC99A005) and 4,188 genes (NEC86) with genes average of 3,945. General features of  
146 these genomes are summarized in Table 2. The draft genome-based phylogeny of *C. neonatale*  
147 isolates enabled the identification of three clusters, revealing clonality among isolates from the  
148 same NICU by following: (1) strain isolated from NICU-1 (LCDC99A005), (2) strain isolated  
149 from NICU-2 (NEC86) and (3) strains isolated from NICU-3 (NEC25, NEC26, NEC32 and C25)  
150 (Figure 2A). Furthermore, core-genome analysis distinguished the same clustering, where the  
151 strict pangenome consisted of 5,150 genes, including 3,157 core-genes (Figure 2B). Predicted  
152 COG categories were homogenous in all *C. neonatale* genomes. The COGs encoding  
153 carbohydrate metabolism and transport, and therefore general functional prediction (G and R),  
154 were over-represented, on the contrary, no treatment or modification of RNA, and therefore the

155 structure and dynamics of chromatin were identified (A and B) (Supplementary Figure S2 and  
156 Table S3).

### 157 **Identification of virulent and unique genes**

158 Comparative analyses identified hemolysin-encoding genes in all strains as follows:  
159 hemolysin A (coverage: 100%, identity: 84% to 92%), two protein sequences encoded for  
160 hemolysin B (coverage: 100%, identity: 44% to 99%) for the first protein and (coverage: 99%,  
161 identity: 76% to 77%) for the second one, hemolysin C (coverage: 96%, identity: 81%) and beta-  
162 hemolysin (coverage: 100%, identity: 92%). We also identified genes encoding for the secretion  
163 of *C. difficile* toxin A and toxin B. Moreover, sequences of *dlt* operon (*dlt A, B, C, D*) were not  
164 detected in all draft genomes of *C. neonatale* (Supplementary Table S4). Unique genes also  
165 existed and mostly represented by hypothetical proteins, as follows: NEC25 (1/4, 25%), NEC26  
166 (77/113, 68%), NEC32 (28/39, 72%), C25 (2/3, 67%), NEC86 (139/212, 65.5%) and  
167 LCDC99A005 (31/59, 52.5%) LCDC99A006 (Supplementary Table S5).

168 **Discussion**

169 Dysbiosis remains a main risk factor inducing the establishment of NEC, by promoting the  
170 translocation of pathogenic bacteria. Specific microorganisms were involved; the predominance  
171 of  $\gamma$ -proteobacteria, generating excessive inflammatory response and irregular colonization by  
172 strictly anaerobic bacteria, including Clostridia [1]. Multidisciplinary approaches suspected the  
173 link between this class and the occurrence of NEC, especially *C. butyricum*, *C. neonatale* and *C.*  
174 *perfringens* [1,21]. To date, *C. butyricum* is the unique *Clostridium* species among the cases of  
175 Clostridia-associated NEC clearly described in correlation with NEC [3,7,22]. However, several  
176 studies have already reported the involvement of *C. neonatale* in NEC disease [1,8]. Using a  
177 culture-based strategy, we observed that the prevalence of *C. neonatale* in stool samples from  
178 patients with NEC was not significantly different from that of controls (respectively 4/88  
179 [4.5%]; vs. 1/71 [1.14%];  $p = 0.26$ ) [7]. During the review process of this previous work, a  
180 reviewer proposed that this discrepancy could be due to the use of heat-shock-based protocol. If  
181 it is supposed to kill all bacteria species except those spore-forming like *C. neonatale*, we could  
182 not exclude that the method skips the isolation of *in vivo* non-spore-form of Clostridia species.  
183 Therefore, we decided to investigate our cohort using *C. neonatale* specific *rpoB*-based qPCR.  
184 With a strong phylogeny and taxonomy index, *rpoB* is a conserved gene whose efficacy for the  
185 identification of fastidious microorganisms directly from samples has been previously  
186 demonstrated [23,24] and which was used for the heterogeneous detection of BoNT producing  
187 Clostridia [25] and *C. butyricum* directly from stool samples [3]. A significant frequency of *C.*  
188 *neonatale* in stools from NEC was detected compared to controls (respectively 34% vs 13%,  $p =$   
189 0.003). In contrast with the study conducted by Rozé *et al.*, in which the authors reported links  
190 between feeding strategies and NEC and the abundance of *C. neonatale* in NEC patients [9], the

191 feeding strategy was herein not associated with the development of NEC. The only correlation  
192 identified was the vaginal delivery mode, a feature already observed in NEC cases [2]. Beside *C.*  
193 *neonatale* and *C. butyricum*, several NEC outbreaks have been described to be associated with  
194 other bacterial species [1,2]. A case report described toxin-producing *C. perfringens*, where NEC  
195 severity was independent from the  $\alpha$ -toxin concentration [26]. Also, nosocomial colonization by  
196 *Klebsiella pneumoniae* type 26 and antibiotic typing was used to specify strain characteristics  
197 [27]. Furthermore, genetically similar clones of *Enterobacter sakazakii* were distinguished from  
198 powdered milk formula and neonates with NEC [28]. Few other bacterial species were suspected  
199 in association with NEC like UPEC and *C. paraputrificum* [4,29].

200 The use of WGS allowed us to further compare and characterize isolates even if the number  
201 of strains and available genomes was limited. This study disclosed genetic similarity between  
202 strains isolated from the same NICU. Through phylogenetic analysis, we identified three  
203 distinct clusters, highlighted by their geographical area of isolation, suggesting the same clone  
204 of *C. neonatale* spreading in the same NICU-3 as we previously observed with *C. butyricum*  
205 [7]. This is in agreement with the discovery of this species as it was firstly isolated from a  
206 Canadian NICU (strains LCDC) and clonality between isolates was proved by PFGE from  
207 stool and blood cultures [8]. Geographic and temporal clustering of NEC were described in  
208 several studies with various etiologies. Temporal clustering was reported by Faustini *et al.* in  
209 diverse NICUs [30]. The similarity between control (C25) and NEC-associated *C. neonatale* in  
210 cluster [3] presupposes the existence of asymptomatic carriage. This same kind of mechanism  
211 was described in case of *C. difficile*-associated pseudomembranous colitis [31] and we  
212 suggested a similar mechanism for *C. butyricum* in neonates [7]. It should be noted that there  
213 appears to be a strong association between *C. neonatale* and neonates/children as it is a species

214 that has never been isolated from adults in our microbiota studies [32]. On the contrary, *C.*  
215 *neonatale* has been identified by sequencing of 16S rRNA in the microbiota of young children  
216 with a strong association of developing asthma [33].

217 If the association of Clostridia with NEC was increasingly reported, the pathogeny of this  
218 disease remains elusive in spite production of toxins is usually suggested, as in the case of *C.*  
219 *difficile* infections (CDI) [33]. Herein, genes encoding for the secretion of bacterial toxins  
220 were predicted, especially hemolysin and *C. difficile* toxin A/B (TcdA/B). These later, is a  
221 leading cause of CDI, where the pathogenic mechanism was the consequence of TcdA  
222 (enterotoxin) and TcdB (cytotoxin) productions inducing colonic tissue damage [25,26]. The  
223 idea of toxin-mediated disease rather than invasive-mediated disease is supported by the work  
224 of Heida *et al.* [34]. Furthermore, hemolysins sequences with highly conserved domain found  
225 in a toxin of *Brachyspira hyodysenteriae* that disclosed cytolytic effect on several cell lines  
226 [13]. Cassir *et al.* identified cytotoxic activity of *C. butyricum* supernatant on Jurkat cells [4].  
227 In conclusion, this study highlights the association between *C. neonatale* and NEC as the  
228 possible existence NEC-associated geographic clones. Further genomic analysis is required on  
229 a larger number of sequenced genomes.

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241 **Conflict of interest**

242 None of the authors have any conflicts of interest to report.

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337

338 **Table 1: Factors associated with the occurrence of necrotizing enterocolitis when compared with control preterm neonates**

Factors	NEC	Controls	Univariate analysis	Multivariate analysis	
	(n=88) No. (%)	(n=71) No. (%)	<i>p</i> value	OR [95% CI]	<i>p</i> 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 ( $\leq$ 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
<i>C. neonatale</i> q-PCR positive	30 (34)	9 (13)	0.003	3.7 [1.6-8.8]	0.003

339 SD = standard deviation; NA = not applicable.

340 **Table 2: Characteristics of *Clostridium neonatale* draft genomes**

<b>Isolate</b>	<b>Location, date of isolation</b>	<b>Sequencing technique</b>	<b>Scaffolds number</b>	<b>Genome length (base pair)</b>	<b>GC content (%)</b>	<b>ORFs number</b>	<b>Accession number</b>
LCDC 99A005	NICU-1, 2000	Mate-pair	3	4,304,459	28.6	3,795	GCA_001458595.1
LCDC 99A006	NICU-1, 2000	NA	58	4,658,596	28.4	4,139	GCA_002553455.1
NEC25	NICU-3, December 2011	Paired-end	3	4,352,344	28.7	3,883	UWJD01000001- 01000003
NEC26	NICU-3, July 2012	Paired-end	3	4,282,644	28.9	3,840	UICU01000001- 01000003
NEC32	NICU-3, December 2012	Paired-end	3	4,321,416	28.7	3,859	UICW01000001- 01000003
NEC86	NICU-2, December 2010	Paired-end, Nanopore	3	4,739,641	28.6	4,188	UICR01000001- 01000003

C25	NICU-3, February 2012	Paired-end	3	4,358,232	28.6	3,913	UICQ01000001- 01000003
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341 ORF: Open-Reading Frame number, NEC: Necrotizing enterocolitis; C25: control; NICU-1: Canada; NICU-2: Marseille, France;

342 NICU-3: Marseille, France, NA: Not available

343 **Figure 1: *In silico* analysis of *Clostridium neonatale rpoB* specific region.** c This analysis was performed on the selected region of

344 151 nucleotides. Phylogenetic analysis showed that the *rpoB* sequence is homogenous among *C. neonatale* and classified it among

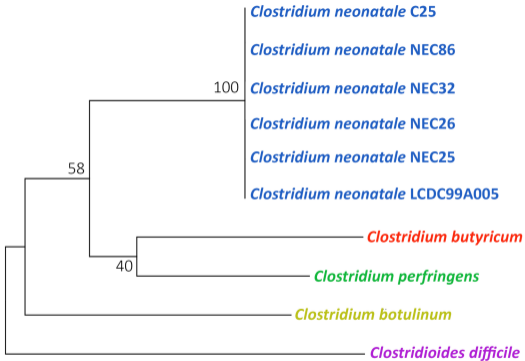
345 *Clostridium* species. *C. difficile* was out grouped.

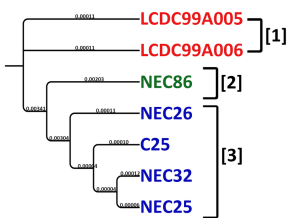
346 NEC: Necrotizing enterocolitis, C25: control.

347 **Figure 2: Relationship of *Clostridium neonatale* strains based on genomic analysis**

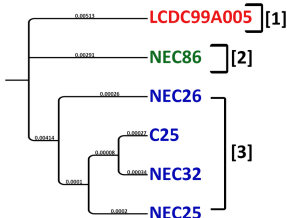
348 (A): phylogenetic tree based on core-genome, (B): phylogenetic tree based on whole-genome alignment, NEC: Necrotizing

349 enterocolitis, C25: control.





(A)



(B)

■ NICU-1 (Canada)  
■ NICU-2 (Marseille)  
■ NICU-3 (Marseille)