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Laurent Ferraris, Johanne Delannoy, Christelle Mazuet, L. Diancourt, Victoria Mesa-Schein, et al.. Clostridium neonatale antimicrobial susceptibility, genetic resistance determinants, and genotyping: a multicentre spatiotemporal retrospective analysis. *Journal of Antimicrobial Chemotherapy*, 2024, 79 (2), pp.271-279. 10.1093/jac/dkad369 . cea-04524814

HAL Id: cea-04524814

<https://cea.hal.science/cea-04524814>

Submitted on 28 Mar 2024

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Clostridium neonatale antimicrobial susceptibility, genetic resistance determinants, and genotyping: a multicentre spatiotemporal retrospective analysis

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Received 4 July 2023; accepted 21 November 2023

Background: *Clostridium neonatale* was isolated during an outbreak of neonatal necrotizing enterocolitis (NEC) in 2002. *C. neonatale* was validated as a new species within the genus *Clostridium sensu stricto* in 2018. In the present study, we evaluated the antimicrobial susceptibility, genetic determinants of resistance, and phylogenetic relationships of a collection of clinical isolates of *C. neonatale*.

Methods: *C. neonatale* strains ($n=68$) were isolated from the stools of preterm neonates who either developed NEC or were asymptomatic carriers of *C. neonatale* in different periods and in different hospitals. Antimicrobial susceptibility was determined by the disc diffusion method. The MICs of clindamycin, cefotaxime and tetracycline were determined. Genetic determinants of resistance were screened by PCR ($n=68$) and WGS ($n=35$). Genotyping of the isolates was performed by MLST.

Results: Antimicrobial resistance was found to clindamycin ($n=24$; 35%), cefotaxime ($n=7$; 10%) and tetracycline ($n=1$; 1%). One clindamycin-resistant isolate carried *erm(B)* by PCR. In addition, one isolate carrying *tet(M)* was tetracycline resistant (MIC = 16 mg/L) and 44 isolates carrying either *tet(O)*, *tet(32)* or *tet(M)* were tetracycline susceptible (MICs < 16 mg/L). MLST showed that ST2 and ST15 were significantly associated with *tet(32)* ($P < 0.0001$) and *tet(O)* ($P < 0.0001$), respectively. From WGS, we identified *aph(3')-IIa* and *bla_{TEM-116}* genes and a *bla_{CBP-1}*-like gene.

Conclusions: *C. neonatale* is susceptible to anti-anaerobic molecules but resistant to clindamycin, cefotaxime and tetracycline. Genes encoding tetracycline ribosomal protection, macrolide-lincosamide-streptogramin B rRNA methyltransferase, aminoglycoside 3'-phosphotransferase and β -lactamases have been identified in genomic regions flanked by mobile genetic elements.

Introduction

Clostridia are ubiquitous and are found in the soil, marine sediments, decaying vegetation, and the intestinal tracts of humans, vertebrates and insects. The genus *Clostridium* has more than 330 validly described species and 12 subspecies.¹ Some species are human and animal pathogens responsible for endogenous or exogenous infections. In particular, *Clostridium botulinum*, *Clostridium tetani*, *Clostridium perfringens* and *Clostridioides difficile* (formerly *Clostridium difficile*) are responsible for human botulism, tetanus, gangrene, antibiotic-associated diarrhoea and food poisoning.² Bacteria of the genus *Clostridium* are important members of human anaerobic gastrointestinal and cervical/vaginal

microbiota. The genus also includes species whose potential pathogenicity and clinical significance are poorly understood.

Clostridium neonatale is a strictly anaerobic, mobile, Gram-positive rod with discernible subterminal spores. It was proposed to be involved in necrotizing enterocolitis (NEC), a devastating gastrointestinal disease with a high morbidity and mortality.³ *C. neonatale* was first isolated from blood cultures of premature neonates (PNs) in 2002 during an outbreak of NEC in a Canadian neonatal ICU (NICU).⁴ At that time, *C. neonatale* was only briefly reported, and its species validation was not published. In 2014, a study combining genetic and phenotypic analyses proposed considering *C. neonatale* as a new species within the genus *Clostridium*.⁵ Finally, in 2018, the name *C. neonatale*

and species validation were published, classifying *C. neonatale* sp. nov. in the *Clostridium* genus cluster I *sensu stricto*.⁶ Within this genus, *C. neonatale* is most closely related to *Clostridium butyricum*.^{5,6} Like *C. neonatale*, *C. butyricum* has been proposed to be involved in NEC development.^{7–11} Since 2002, *C. neonatale* has been repeatedly associated with NEC in multicentre and regional studies, and a case report has been published.^{9,12,13} In a preterm piglet animal model, both *C. neonatale* and *C. butyricum* were significantly overrepresented in ileal mucosal samples from animals with NEC.¹⁴ These data support the pathogenic role of *C. neonatale*, but the exact mechanisms by which *C. neonatale* interacts with the host and participates in the development of NEC remains to be elucidated. Of note, *C. neonatale* has also been isolated from the commensal gut microbiota of neonates without NEC, suggesting possible asymptomatic carriage.^{15–17}

The confusing status of *C. neonatale* until its recent formal classification may explain the lack of data on this species in terms of clinical significance and epidemiology. This may have contributed to the misidentification of *C. neonatale* in previous studies, including those on NEC. As a result, very little is known about the antimicrobial susceptibility patterns and genetic resistance determinants of *C. neonatale*. Of note, PNs are often treated with broad-spectrum antibiotics, and NEC treatment includes the administration of broad-spectrum antibiotics, including β -lactams, clindamycin or metronidazole.¹⁸ Therefore, the aim of this study was to report the antimicrobial susceptibility profiles using the disc diffusion method of *C. neonatale* strains ($n=68$) isolated from stool samples of neonates born preterm who developed NEC or were asymptomatic carriers of *C. neonatale*. Because resistance has been previously reported for *Clostridium* spp., we also determined MICs of clindamycin, tetracycline and cefotaxime. In addition, all isolates were screened by PCR for *tet* and *erm* genetic resistance determinants. The phylogenetic relationships of all *C. neonatale* isolates were analysed by MLST at different periods and in different hospitals. For a subset of the isolates ($n=35$), WGS analysis allowed identification of putative genetic determinants and comparison of specific genetic regions.

Methods

Strain isolation and identification

The 68 *C. neonatale* isolates used in this study are part of our laboratory collection (Laboratoire de Microbiologie, UMR-S1139, Faculté de Pharmacie, Université Paris Cité). Strains were isolated from the stool samples from different infants born preterm enrolled in three different cohorts (Table S1, available as [Supplementary data](#) at JAC Online). Briefly, the single-centre PREMAFLORA cohort (ANR-07-PNRA-007) enrolled infants of gestational age less than 37 weeks hospitalized in a French NICU (2008–09).¹⁹ Both the EPIFLORE (2011) and CloNEC (2015–16) cohorts were multicentre NEC case–control studies involving 12 French NICUs, enrolling preterm neonates with gestational age less than 32 weeks.^{9,20} No NEC outbreak was reported during the inclusion periods. These studies were conducted in accordance with the relevant French guidelines and regulations, and informed consent was obtained from the parents of all enrolled children.

Briefly, for *C. neonatale* isolation, stool samples were crushed in brain-heart infusion broth using an Ultra-Turrax T25 (Fisher-Bioblock, Illkirch, France), diluted in peptone water, and spread on sulphite-polymyxin-milk selective agar medium at 10^{-2} , 10^{-4} and 10^{-6} dilutions using WASP

apparatus (Don Whitley Scientific, UK).²¹ Media were incubated for 48 h at 37°C under anaerobic conditions ($H_2:CO_2:N_2$, 10:10:80, v/v/v) in an A35 anaerobic workstation (Don Whitley Scientific). Strains were conserved in brain heart infusion medium with 20% (v/v) glycerol at -80°C . Species identification was performed using a MALDI-TOF MS Microflex spectrometer (Bruker Daltonics S.A.) and PCR amplification and sequencing of the 16S rDNA gene, as previously described.⁵ A total of 68 *C. neonatale* isolates were included in the present study, 17 of which were obtained from cases of NEC and 51 from asymptomatic carriers (Table S1). The reference strain 250.09 (NML 99A005^T=ATCC BAA-265^T=CCUG 46077^T) was included among the 68 isolates used in the present study.⁵ Bacterial liquid cultures were performed in TGYH broth (tryptone 30 g/L, glucose 5 g/L, yeast extract 20 g/L and haemin 5 mg/L) for 48 h at 37°C under anaerobic conditions.

MLST

The housekeeping genes *ddl*, *dnak*, *groEL*, *gyrA*, *rpoB* and *CTPs* were used for MLST.⁵ For 33 isolates, gene sequences were obtained after PCR amplification and Sanger sequencing of both strands (GENEWIZ, Leipzig, Germany). For 35 isolates, gene sequences were obtained by *in silico* analysis of *de novo* scaffold genomes. Briefly, genomic DNA extraction was performed on 24 h bacterial liquid cultures using the DNA Easy UltraClean microbial kit (QIAGEN, Courtaboeuf, France). Sequencing of the 35 *C. neonatale* genomes was performed by the Biomix Sequencing Platform at the Institut Pasteur (Paris, France) using the Illumina Nextera XT DNA Library Prep Kit and the NextSeq 500 system sequencer. *De novo* assembly of the 35 genomes was performed as previously described.²² Comparison of each housekeeping gene sequence was performed using the NCBI multisequence advanced BLAST software.²³ An allele number is assigned to each unique sequence. An ST is assigned to each strain based on the combination of allele numbers for the selected loci that allow the strains to be classified. Clonal complexes, defined as a set of strains that differ by no more than one allele at a single locus, were determined using the START2 based upon related sequence types (BURST) algorithm.²⁴ A neighbour-joining tree based on the STs profiles was constructed using PHYLOViZ v.2.0²⁵ applying the Hamming distance and the Saitou–Nei branch-length minimization criteria. The tree was edited using the iTOL v.6.5.7 tool.²⁶

Antimicrobial susceptibility

Antimicrobial susceptibility testing was performed using the disc diffusion method on Brucella agar medium supplemented with 5% (v/v) sheep blood and 1 $\mu\text{g}/\text{mL}$ vitamin K1 according to the EUCAST recommendations.²⁷ The antibiotic discs tested (bioMérieux, Marcy l'Étoile, France) included amoxicillin, amoxicillin/clavulanic acid, piperacillin, piperacillin/tazobactam, ertapenem, imipenem, cefoxitin, tigecycline, chloramphenicol, moxifloxacin, metronidazole, linezolid and vancomycin. The MIC determinations for clindamycin, tetracycline and cefotaxime were performed using ETEST strips according to the manufacturer's instructions (bioMérieux) due to previously reported resistance of *Clostridium* spp. The breakpoints used in our study are those of the 'Comité de l'Antibiogramme de la Société Française de Microbiologie' (CA-SFM) version 2013 for anaerobic bacteria,²⁸ which have not been updated since the adoption of the EUCAST methods. Detection of β -lactamase production was performed on the isolates resistant to cefotaxime using the nitrocefin chromogenic assay, as recommended by the manufacturer (Sigma–Aldrich Chimie, Saint-Quentin-Fallavier, France).

tet and *erm* genes

Genomic DNA from the 68 isolates was purified (InstaGene kit, Bio-Rad) and used as a template for the PCR amplification of the *tet*(M), *tet*(W), *tet*(O), *tet*(Q), *erm*(B), *erm*(Q) and *erm*(F) genes, as previously described.²⁹

Sanger sequencing of *tet* and *erm* amplicons was performed by GENEWIZ (Germany), and sequences were analysed using the NCBI BLAST software.²³ The 35 *de novo* scaffold-assembled genomes were used to compare the genetic environment of the antimicrobial resistance genes using the MicroScope platform v3.14.3 (last accessed July 2021)³⁰ and visualized using Easyfig.³¹ The ABRicate v1.0.1 software (<https://github.com/tseemann/abricate>) and the MEGARes 2.0³² and CARD³³ databases were used for antimicrobial resistance gene prediction of the 35 *de novo* scaffold-assembled genomes.

Nucleotide sequences

MLST new allele sequences were deposited in the GenBank database under the accession numbers MT774173 and MT774174 (*CTPs* alleles), MT783691 to MT783695 (*ddl* alleles), MT783696 (*dnaK* alleles) and MT774172 (*gyrA* alleles) (Table S2). Nucleotide sequences of the *tet*(O), *tet*(M), *tet*(32) and *erm*(B) genes were deposited in the GenBank database under the accession numbers OL555789, OL555791, OL555790 and OL555788, respectively (Table S2). The 35 bacterial genomes used in the present study are available at the European Nucleotide Archive database under the accession numbers ERS13471567, ERS13471572, ERS13471575 to ERS13471586, ERS13471588 to ERS13471605, and ERS7257048 to ERS7257050 (Table S2).

Statistical analysis

XLSTAT version 2014.5.03 was used for statistical analysis. Fisher's exact test was used to determine non-random associations between two categorical variables. The Mann-Whitney *U*-test was used to compare the observations between two groups. Significance was set at $P < 0.05$.

Results and discussion

Antimicrobial susceptibility

The disc diffusion method data showed that the 68 *C. neonatale* isolates, including the reference strain 250.09, were susceptible to amoxicillin, amoxicillin/clavulanic acid, cefoxitin, piperacillin, piperacillin/tazobactam, ertapenem, imipenem, chloramphenicol, tigecycline, moxifloxacin, metronidazole, linezolid and vancomycin. The susceptibility of *C. neonatale* was similar to that reported for *C. butyricum* strains isolated from stool samples of preterm infants^{3,29} and for other *Clostridium* spp.^{34–36}

Resistance to clindamycin, tetracycline and β -lactams has been reported for *Clostridium* spp.^{3,22,35–38} A retrospective study analysing the antimicrobial susceptibility of gas gangrene-forming *Clostridium* spp. over a 10 year period in Hungary reported resistance of up to 6.8% and 8.5% to penicillin and

clindamycin, respectively.³⁷ Similar levels of resistance have been observed in *Clostridium* spp. isolated from bloodstream infections.³⁸ We have previously reported resistance of *Clostridium* spp. to clindamycin, tetracycline and cefotaxime.^{3,29} In addition, we reported the resistance of *C. neonatale* LF22 to tetracycline (MIC = 12 mg/L) in 2022.²² Therefore, in the present study, we performed MIC determinations for clindamycin, tetracycline and cefotaxime. Some *C. neonatale* isolates were resistant to clindamycin ($n = 24$; 35%), the third-generation cephalosporin cefotaxime ($n = 7$; 10%) and/or tetracycline ($n = 1$; 1%) (Table 1). The MIC₉₀ of clindamycin (12 mg/L) corresponded to resistance (MIC > 4 mg/L) (Table 1).

Resistance to β -lactams has been reported for some *Clostridium* spp.,³⁹ and particularly for *C. difficile*, with resistance rates up to 95%.³⁵ However, the underlying mechanisms of resistance are currently unknown. β -Lactamase-mediated resistance has been reported for *C. butyricum*, *Clostridium clostridioforme*, *Clostridium ramosum*, *C. difficile* and *Clostridium botulinum*.^{40,41–44} In the present study, we observed that some *C. neonatale* isolates were resistant to cefotaxime, but all tested negative when the nitrocefin chromogenic assay was used to assess potential β -lactamase production. Other mechanisms of resistance to β -lactams have also been proposed for *Clostridium* spp., but they are less studied and far less common.³⁹

Concerning tetracycline and clindamycin, in 2022, we identified a *tet*(W/N/W) gene encoding a tetracycline resistance ribosomal protection protein and an *erm*(Q) gene encoding a 23S rRNA methyltransferase in two different *C. neonatale* isolates.²² In this study, we observed some resistance to tetracycline and clindamycin. Therefore, all isolates were screened for the presence of the *tet* and *erm* genes, which are the most frequently reported resistance genes to these antibiotics.^{45,46}

tet genes

In the present study, a total of 45 (66%) isolates were found to carry a *tet* gene by PCR (Figure 1 and Table S1). One isolate, E15, was tetracycline resistant (MIC = 16 mg/L) and 44 were susceptible (MICs ≤ 8 mg/L) (Table 1 and Table S1). However, the 44 tetracycline-susceptible isolates had MICs 32 to 64 times higher (ranging from 1 to 8 mg/L) than those of the 23 isolates without *tet* (MICs ≤ 0.125 mg) (Tables 1 and Table S1). These data suggest that the *tet* genes confer different levels of resistance to tetracycline. For all 45 isolates, the 1200 bp PCR products were sequenced,

Table 1. MICs of tetracycline, clindamycin and cefotaxime for *C. neonatale* isolates

Antimicrobial agents	$\leq 0.125^a$	No. of strains for which the antimicrobial agent MIC (mg/L) was:											MIC (mg/L)		
		1	1.5	2	3	4	6	8	12	16	32	>32	MIC ₅₀	MIC ₉₀	
Tetracycline	23 ^b	2	7	13	3	8	7	4		1				2	6
Clindamycin	4	4		18	2	16 ^b	9	8	5	2				4	12
Cefotaxime								1		23	37 ^b	7		32	32

Clinical resistance breakpoints: clindamycin MICs > 4 mg/L; cefotaxime MICs > 32 mg/L; tetracycline MICs > 8 mg/L.²⁸ Values in bold correspond to resistance.

^aStrains without *tet* gene.

^bMICs for the reference strain 250.09 (=LDCDC 99A005^T) as reported by Alfa *et al.*⁴

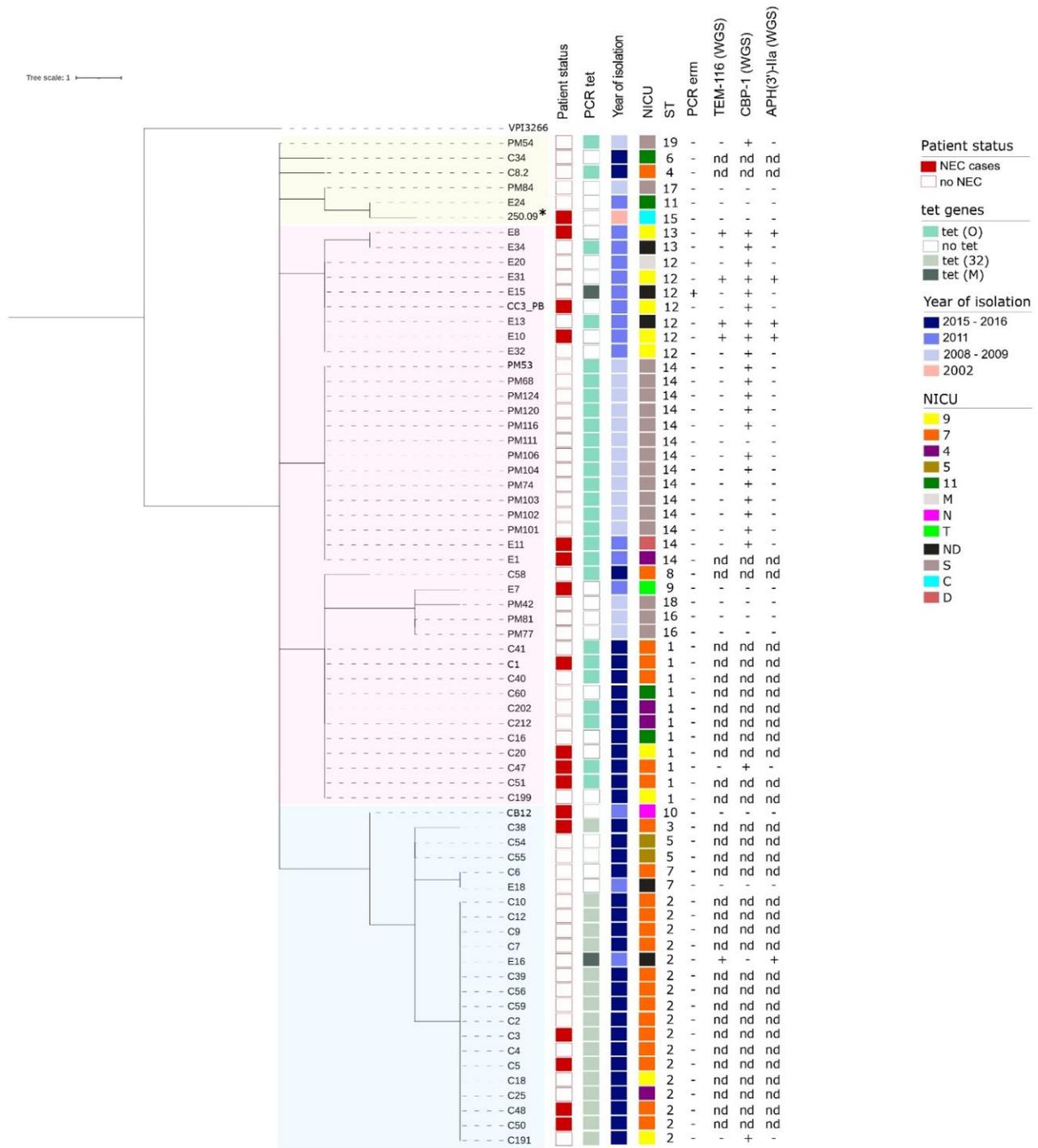


Figure 1. Neighbour-joining phylogenetic tree based on the ST profiles of the 68 *C. neonatae* isolates determined by START2 software and created using GrapeTree software v.1.5.0. *C. butyricum* VPI3266¹ was used as an outgroup. Numbers to the right of the tree refer to the strain ID. Clonal complexes are colour-coded yellow/pink/blue. **C. neonatae* 250.09 reference strain. nd: not determined.

and the Blast results were consistent with the *tet* genes. The scaffold-assembled genome of isolate E15 allowed identification of a 1920 bp coding sequence corresponding to the *tet(M)* gene of *C. difficile* (accession no. JN846703.1) and *Streptococcus suis*

(accession no. MT383668) (Figure 1a). Of the 44 susceptible isolates with a *tet* gene, 26 (59%), 17 (39%) and 1 (2%) carried either a *tet(O)*, *tet(32)* or *tet(M)* gene, respectively (Figure 1 and Table S1). The gene nucleotide sequences were identical to

tet(O) of *Campylobacter jejuni* (accession no. M18896.2), *tet(32)* of *Streptococcus salivarius* (accession no. EF626943.1) and *tet(M)* of *C. difficile* (accession no. JN846703.1). Compared with other clostridia, the most widespread *tet* gene is *tet(M)* for *C. difficile*^{47,48} and *C. perfringens*⁴⁹ while *tet(O)*, *tet(W)* or *tet(32)* are less frequently reported.^{47,48} Statistically, isolates carrying *tet(O)* had significantly higher MICs compared with those carrying *tet(32)* ($P < 0.0001$). In both Gram-positive and Gram-negative bacteria, *tet* genes are often found flanked by or located in genomic regions with mobile genetic elements.^{45,50} In the present study, *tet(M)* was found in the resistant isolate E15, but also in the susceptible isolate E16. Interestingly, *tet(M)* was located in different genetic regions (Figure 2a). The genetic environment of *tet(M)* compared with the reference strain 250.09 genome showed that the E15 and E16 genetic regions were characterized by the presence of integrative and conjugative elements (ICEs) associated with the ICEBs1 genes *conQ*, *cwlT*, *conE* and *nick* (Figure 2a).^{51,52} These genes are related to genes found in Tn916 and other Gram-positive conjugative elements that have been reported to have different genetic organizations and carry different *tet* alleles, as well as other antibiotic resistance determinants.⁵⁰ The *tet(M)* gene of both the E15 and E16 isolates is located on a transposon that shares 99.35% nucleotide identity with the Tn916 transposon of *Enterococcus faecalis* DS16 (accession no. U09422.1). Comparison of the regions surrounding *tet(O)* and *tet(32)* from two representative tetracycline-susceptible isolates (E11 and C191, respectively) showed that the genes were located in distinct genomic locations associated with genes encoding putative Tn1549-like proteins (Figure 2b and c). The genetic region encompassing *tet(O)* was absent from the genome of the reference strain 250.09, as well as E7, E18, E24, C6, C55, CB12, PM42, PM77, PM81 and PM84 isolates. Altogether, these data suggest deletion and recombination events consistent with the previously reported genetic plasticity of *C. neonatale*, which is characterized by multiple mobile genetic elements, including genomic islands, insertion sequences, and predicted intact prophage signatures.²²

erm genes

In the present study, of the 68 isolates, only isolate E15 was found to carry an *erm* gene by PCR, although 24 were found to be clindamycin resistant (Table S1). The PCR product was sequenced and the Blast results matched with the *erm(B)* gene of *C. difficile* 630 (accession no. CP010905) and *Staphylococcus intermedius* (accession no. AF299292). This was confirmed by the E15 genome analysis (Figure 3). The genetic region encompassing *erm(B)* was absent from the genome of the reference strain 250.09. A peculiar Tn916-like element containing both *tet(M)* and *erm(B)* has previously been reported in a clinical isolate of *C. difficile*.⁴⁶ In the present study, the *erm(B)* genetic environment was characterized by the presence of genes annotated to encode putative conjugative and Tn916-like proteins (Figure 3). Interestingly, isolate E15, carrying *erm(B)*, also carries a *tet(M)* gene, and both genes are located in different genomic regions (Figures 2a and 3).

Previously, some studies have reported resistance to clindamycin in *C. butyricum* and *C. difficile* isolates that do not carry *erm* genes.^{29,46} It has been proposed that alternative resistance mechanisms such as target site mutations, efflux pumps, or

enzymatic antibiotic inactivation may be involved, but this requires further investigation.

Other genetic resistance determinants

ICEs belonging to the Tn916-like family have been described in several bacterial genera and are responsible for the spread of resistance to several classes of antimicrobial drugs.^{50,53} Identifying *tet* and *erm* genes in genomic regions associated with ICEs raised the question of whether other potential resistance genes might be present in the sequenced genomes, independently of the antimicrobial phenotype of the isolates. Among the 35 isolates for which WGS was available, we identified different genes encoding a putative *bla*_{TEM-116} class A β-lactamase (accession no. U36911.1) ($n=5$ genomes), a class A β-lactamase gene with 81.74% nucleotide identity to *bla*_{CBP-1} (accession no. KP718480.1) ($n=21$ genomes) and a putative aminoglycoside *O*-phosphotransferase *aph(3')-IIa* (accession no. V00618.1) ($n=5$ genomes) (Figure 1 and Table S1). The three genes were identified simultaneously in the genomes of E8, E31, E13 and E10 (Figure 1 and Table S1). The E16 genome carries both the *aph(3')-IIa* and the *bla*_{TEM-116} genes (Figure 1 and Table S1). The E15 genome carries the *bla*_{CBP-1}-like gene (Figure 1 and Table S1). This suggests the possibility of other transposition events and is consistent with the identification of genes encoding putative proteins associated with other transposons such as Tn5397 and Tn1549 (Figure 2b and c). A comprehensive analysis of the genomes needs to be performed to more specifically identify regions lacking the transposase gene, insertion sequences and transposon elements. Identification of these elements will provide essential features of *C. neonatale* genetic rearrangements.

We have mentioned that β-lactamase-mediated resistance has been reported for *Clostridium* spp.^{40,41-44} In the present study, we observed that *C. neonatale* isolates were resistant to cefotaxime, but tested negative using the nitrocefin chromogenic assay to assess potential β-lactamase production. Whether the identified β-lactamase genes are expressed and contribute to *C. neonatale* cefotaxime resistance and more generally to β-lactam resistance needs to be addressed.

MLST

Seventeen isolates were from the single-centre study PREMAFLORA during 2008–09, 16 from the nationwide multicentre study EPIFLORE in 2011, and 34 from the nationwide multicentre study CLoSNEC during 2015–16 (Figure 1 and Table S1). With the exception of 2008 (single-centre cohort), the isolates originated from eleven different NICUs, representing five French regions (Figure 1 and Figure S1). The genetic relatedness and evolution of the isolates with or without resistance genes at different periods and in different hospitals are presented as a phylogenetic tree in Figure 1. The 68 isolates belonged to 19 different STs, mainly ST2 (25%), ST15 (21%), ST1 (16%) and ST12 (10%) (Figure 1 and Table S1). The reference strain 250.09 belongs to ST16. According to the BURST analysis, the 19 STs belonged to three clonal complexes (each ST matched at least one other ST in the group at four or more MLST loci) (Figure 1). The phylogenetic tree showed that some isolates were organized in tight (near-clonal) groups and were associated with isolation from the same period or cohort, suggesting possible clone spread

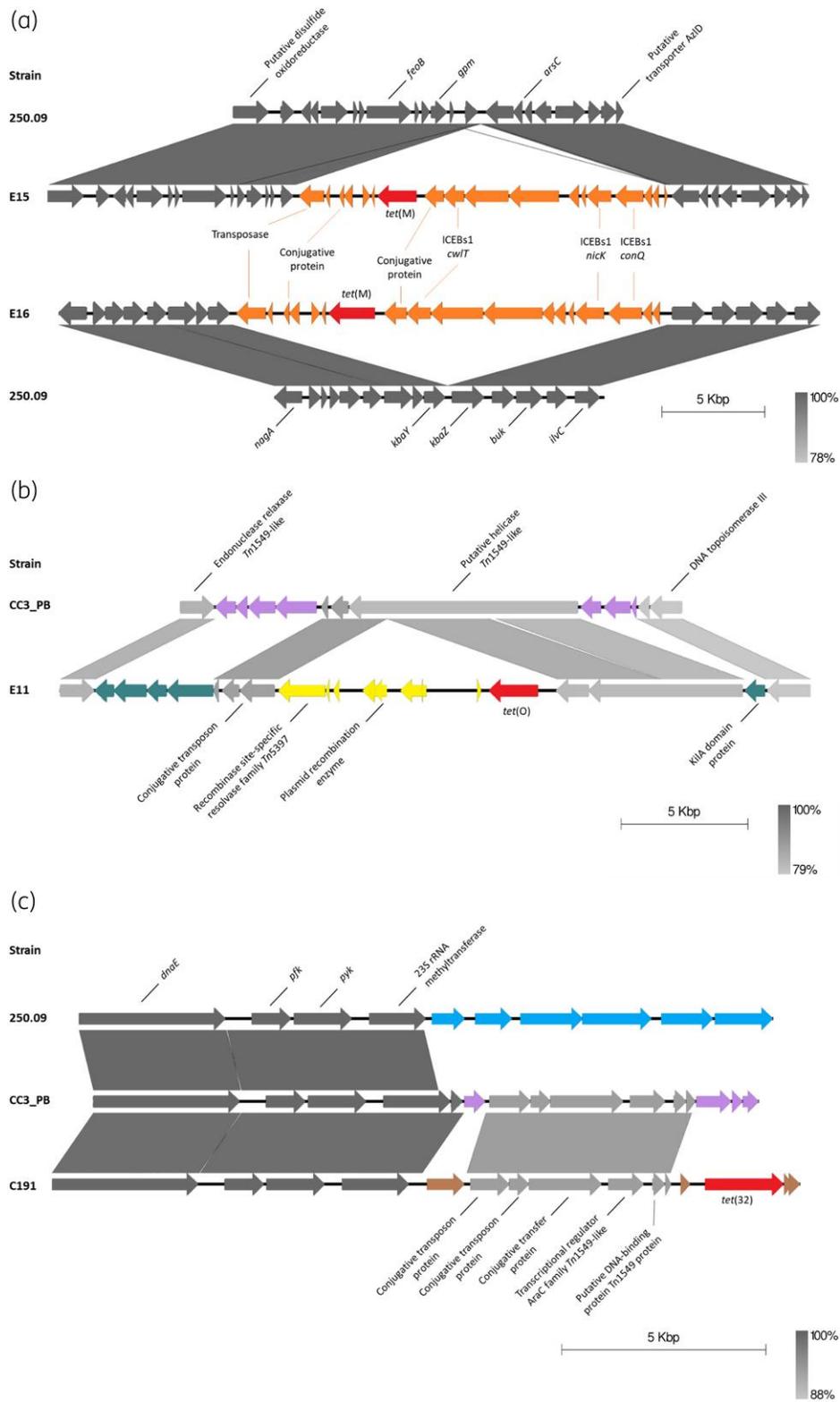


Figure 2. Genomic environment of the *tet* genes. (a) Genomic region comparison of *tet(M)* between the reference strain 205.09 and the E15 and E16 strains. (b) Genomic region comparison of *tet(O)* between CC3_PB and E11 strains. Since the genetic region encompassing *tet(O)* is absent from the reference strain 250.09 genome, we used the CC3_PB genome for comparison. (c) Genomic region comparison of *tet(32)* between the reference strain 250.09, CC3_PB and C191. Comparison was performed using the MicroScope platform v3.14.3 (last accessed July 2021)³⁰ and visualized using Easyfig.³¹ Strains 250.09, E16, CC3_PB, E11 and C191 are susceptible to tetracycline (MIC \leq 8 mg/L). Strain E15 is tetracycline resistant (MIC = 16 mg/L).

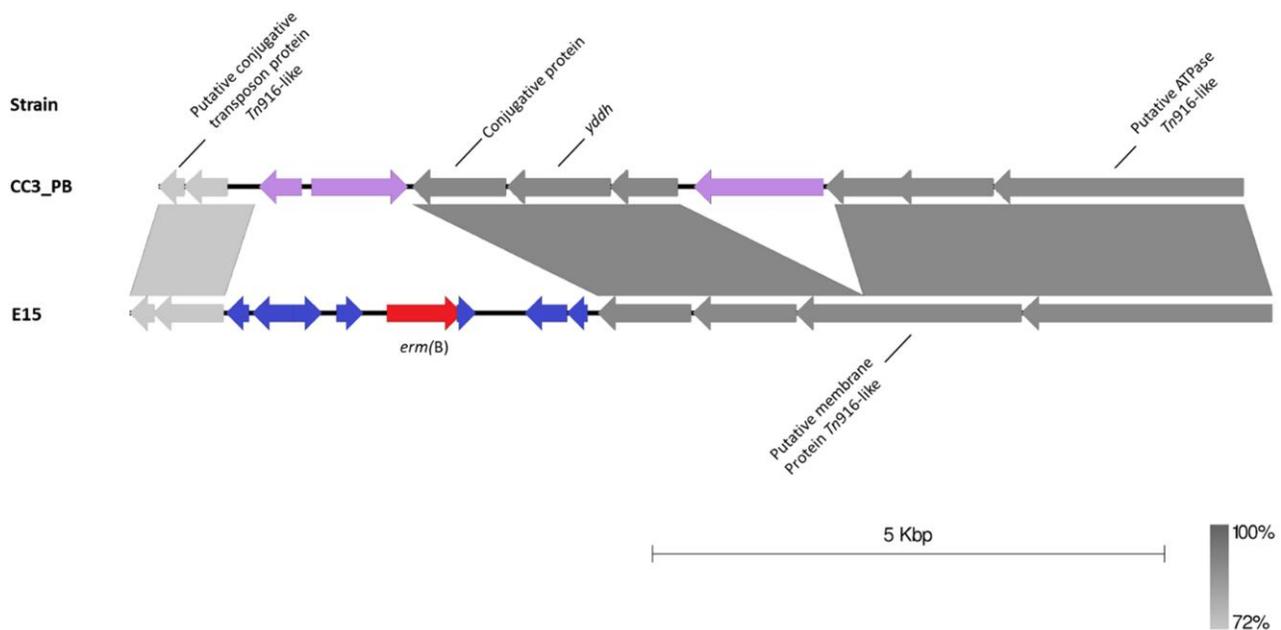


Figure 3. Genomic region comparison of *erm(B)* between CC3_PB and E15. Since the genetic region encompassing *erm(B)* is absent from the reference strain 250.09 genome, we used the CC3_PB genome for comparison. Comparison was performed using the MicroScope platform v3.14.3 (last accessed July 2021)³⁰ and visualized using Easyfig.³¹ Strain CC3_PB is susceptible to clindamycin (MIC = 1 mg/L).²² Strain E15 is resistant to clindamycin (MIC = 8 mg/L).

(Figure 1). However, clonal strains also clustered independently of periods or cohorts. Considering the time period of strain isolation and *tet* carriage, *tet(O)* was identified in 2008, 2011 and 2015–16 (Figure 1 and Table S1). Isolates carrying *tet(O)* were distributed among seven different STs, most frequently ST15 (54%) and ST1 (27%) (Figure 1, Tables S1 and S2). The two isolates carrying *tet(M)* were isolated in 2011 and belonged to ST2 and ST12 (Figure 1). The ST2 (94%) and ST3 (6%) included all 17 isolates carrying *tet(32)*, which were only isolated during 2015–16 (Figure 1, Tables S1 and S2). These data suggest that the number of *tet(O)*-carrying isolates decreased over time, while the number of *tet(32)*-carrying isolates increased. This is supported by the localization of the *tet* genes in genomic regions characterized by the presence of mobile genetic elements, which favour recombination events. Statistically, ST2 and ST15 were significantly associated with *tet(32)* ($P < 0.0001$) and *tet(O)* ($P < 0.0001$), respectively. No significant ST association was observed when considering isolate resistance to clindamycin, resistance to cefotaxime, clonal complex distribution, or NEC status ($P > 0.25$).

Previously, the emergence of *Streptococcus agalactiae* (Group B *Streptococcus*) as a human pathogen corresponded to the selection and dissemination of a few clones whose expansion was associated with tetracycline resistance, particularly those carrying *tet(M)*.⁵⁴ In the present study, there were no statistical differences when considering *tet* carriage and isolate origin with respect to NEC status ($P = 0.24$).

Conclusions

Recent changes in antibiotic resistance among anaerobic bacteria, including *Clostridium* spp.,⁵⁵ represent a potential

threat.⁵⁶ Therefore, it is important to monitor the resistance patterns of anaerobes. Although the retrospective nature of this study may be a limitation, this work took advantage of a unique large collection of clinical isolates obtained from different hospitals and at different periods and showed that *C. neonatale* isolates are susceptible to anti-anaerobe antimicrobial agents. Some isolates were resistant to clindamycin, tetracycline and cefotaxime. Interestingly, by PCR, we identified *tet(M)*, *tet(O)* and *tet(32)* encoding tetracycline ribosomal protection proteins and a macrolide-lincosamide-streptogramin B rRNA methyltransferase *erm(B)* gene, all associated with mobile genetic elements. By WGS, we identified additional putative β -lactamase and aminoglycoside *O*-phosphotransferase resistance genes. Further work is needed to determine the exact relationship between these genetic determinants and phenotypic antimicrobial susceptibility/resistance levels. In particular, work is in progress to sequence a larger collection of *C. neonatale* genomes to allow a more detailed comparison and analysis of these genomic regions containing antimicrobial resistance determinants. In addition, further studies are needed to determine the possibility of horizontal transfer of these genes. To the best of our knowledge, this study provides the first phenotypic and genetic antibiotic resistance data on *C. neonatale*, a potential opportunistic pathogen associated with NEC.

Acknowledgements

We would like to thank the PREMAFLORA, EPIFLORE and CLoSNEC clinicians and participants.

Funding

This work was supported by the Agence Nationale de la Recherche (ANR-07-PNRA-007, ANR-12-BSV3-0025 and ANR-13-PRTS-0018).

Transparency declarations

All authors: none to declare.

Supplementary data

Figure S1 and Tables S1 and S2 are available as [Supplementary data](#) at JAC Online.

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