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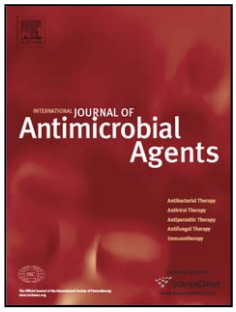

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Antimicrobial susceptibility and resistance determinants of *Clostridium butyricum* isolates from preterm infants

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ABSTRACT

This study reports the antibiotic susceptibility and genetic resistance determinants of 39 *Clostridium butyricum* strains isolated from the faeces of preterm infants as well as one reference strain. Results showed that all the strains were susceptible to cefoxitin, imipenem, vancomycin, tigecycline, metronidazole, chloramphenicol and linezolid. Resistance was observed to clindamycin (100%), penicillin G, amoxicillin and piperacillin (15%), tetracycline (7.5%) and erythromycin (5%). Investigation of the genetic basis of the observed resistance phenotypes showed that resistance to penicillin was due to β-lactamase activity and that resistance to tetracycline involved *tet*(O) or *tet*(O/32/O) homologue genes. Clindamycin and erythromycin resistance may involve another genetic determinant, different from those commonly described for clostridia.

1. Introduction

Preterm infants have delayed bacterial colonisation compared with full-term infants, leading to microbiota imbalance with overgrowth of potentially pathogenic bacteria. This dysbiosis is a risk factor for the onset of gastrointestinal diseases in this target population [1]. For instance, compared with term infants, premature infants at risk of necrotizing enterocolitis (NEC) have a paucity of bacterial species and/or delayed onset of bacterial gut colonisation [2].

Clostridia are among the anaerobes that are part of the indigenous intestinal microbiota of humans. These commensal spore-forming Gram-positive rods belong to the species isolated from the normal neonatal gut bacterial community of full-term [3] and preterm infants [4,5]. In preterm infant gut microbiota, occurrences of clostridia, in particular *Clostridium butyricum*, have been described [4,5]. Clostridial colonisation has been linked to a higher risk of NEC [6], a devastating gastrointestinal disease with high morbidity and mortality, and several investigations have supported the role of clostridial species in NEC pathogenesis [6–10], although other bacterial species have also been implicated [1,11]. Indeed, high production of metabolites through colonic bacterial fermentation is thought to be responsible for the onset of digestive lesions, i.e. gas cysts, haemorrhagic lesions and necrosis, as shown in animal models of NEC [11–13]. Despite the similarities of NEC to clostridial infection, only a few studies have employed anaerobic culture techniques for isolation, identification and characterisation of clostridial strains routinely. Little information is therefore available on this species. In particular, data on its susceptibility to antibiotics are very scarce and relate to few strains, although perinatal antibiotic treatment is very frequent in preterm infants. The aim of this study was to perform and report the first

survey of the antimicrobial susceptibility patterns and genetic resistance determinants of *C. butyricum* isolated from preterm infant faeces.

2. Material and methods

2.1. Isolates and strain identification

Among 102 premature infants screened from four different French hospitals, 39 *C. butyricum* strains were isolated (from 2004 to 2009). Among the 39 strains, 3 were isolated from three different neonates with NEC from the different hospitals.

Strain isolation was as performed as follows. Faecal samples were crushed in brain–hearth infusion broth using an Ultra-Turrax T25 (Fisher-Bioblock, Illkirch, France) and diluted in peptone water and then 10^{-2}, 10^{-4} and 10^{-6} dilutions were spread using a WASP apparatus (AES Chemunex, Bruz, France) on clostridia sulphite–polymyxin–milk selective medium and incubated for 48 h at 37 °C in an anaerobic chamber (AES Chemunex) under anaerobic gas phase (H_2:CO_2:N_2, 10:10:80, v/v/v). Colonies suspected as being clostridia on the basis of cellular morphology and Gram staining were identified using Rapid ID 32A strips (bioMérieux, Marcy l'Etoile, France). Identification was confirmed by partial sequencing of the 16S rRNA gene, which was amplified by polymerase chain reaction (PCR) using primers LPW58 (5'-AGGCCCGGGAACGTATTCAC-3') and LPW81 (5'-TGGCGAACGGGTGAGTAA-3'). Reference strain *C. butyricum* ATCC 19398 was included in the study. Liquid cultures were performed in TGYH broth (tryptone 30 g/L, glucose 5 g/L, yeast extract 20 g/L and hemin 5 mg/L) for 24 h at 37 °C in an anaerobic chamber (AES Chemunex).

2.2. Minimal inhibitory concentration (MIC) determination

MICs for penicillin G, amoxicillin, cefoxitin, piperacillin, imipenem, vancomycin, tetracycline, tigecycline, erythromycin, clindamycin, ofloxacin, metronidazole, chloramphenicol and linezolid were determined using the agar dilution method on *Brucella* agar medium supplemented with 0.5% sheep blood. An inoculum was prepared for each strain by suspending cells from a plate in TGYH broth to achieve a turbidity equivalent to that of a 0.5 McFarland standard (3×10^5 cells/mL) and the inoculum was delivered by a Steers replicator onto agar plates. Resistant and susceptible strains were characterised following Clinical and Laboratory Standards Institute (CLSI) breakpoints [14].

2.3. PCR amplifications

Purified genomic DNA of all 40 strains (39 clinical strains and 1 reference strain) was used as a template for PCR amplification of the protection ribosomal genes *tet*(M), *tet*(W), *tet*(O) and *tet*(Q), the efflux pump genes *tet*(K) and *tet*(L), and the *C. butyricum* chromosomal efflux pump *tet*(P) (GenBank accession no. EDT76835.1), rRNA methylases genes *erm*(B), *erm*(Q) and *erm*(F), and *lmrB* (accession number no. EDT76011) (Table 1). The PCR mixture was composed of 1 μM of each primer, 5% dimethyl sulphoxide (DMSO), each deoxynucleotide triphosphate (dNTP) at a concentration of 250 μM in 1× PCR buffer and 1.25 U of recombinant DNA polymerase (Invitrogen, Illkirch, France) in a final volume of 25 μL. The PCR programme was 4 min at 95 °C, followed by 30 cycles of 30 s at 94 °C, 30 s at 50 °C and 90 s at 72 °C, and a 5 min final extension at 72 °C. When PCR products where obtained for *tet*, *erm* or *lmrB* genes they were sequenced to confirm their identity (Genome Express SA, Meylan, France). Sequences were analysed using the

BLAST Align program available from the National Center for Biotechnology Information (NCBI).

2.4. tet(P) and lmrB reverse transcription polymerase chain reaction (RT-PCR)

After 16 h of growth in TGYH broth, 0.5 mL of bacterial culture was mixed with 1 mL of RNAprotect (QIAGEN, Courtaboeuf, France). Total RNA was isolated using an RNeasy Mini Kit (QIAGEN). Following extraction, DNA contamination was removed by RNase-free DNase (QIAGEN) digestion for 30 min at 37 °C. Total RNA concentration and purity were determined using a NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific, Villebon-sur-Yvette, France) and total RNA was stored at –80 °C. RT-PCR was performed using a SuperScript® III RT Kit (Invitrogen) with 1 μg of total RNA. The primer pairs TetP-ShrtF/TetP-ShrtR and LmrB-ShrtF/LmrB-ShrtR (Table 1) were used for cDNA amplification. The *C. butyricum* housekeeping gene *recA* (accession no. EDT76977) was used as a reference, and cDNA amplification was performed using primers RecA-F/RecA-R (Table 1). One microlitre of the resulting cDNA was amplified with *tet*(P) and *lmrB* primers (Table 1).

2.5. Nucleotide sequence accession numbers

Nucleotide sequences of the potential *tet*(O/32/O) gene homologue and *tet*(O) genes were deposited in the GenBank database under the accession nos. GQ240299, GQ240297 and GQ240298, respectively.

3. Results and discussion

3.1. Susceptibility levels

Non-*perfringens Clostridium* spp. have been reported to be susceptible to penicillins, imipenem and metronidazole, whereas resistance occurred to cefoxitin and clindamycin [15,16]. With regard to *C. butyricum*, no comparative data were available because its susceptibility levels were included among the *Clostridium* spp. group. In this study, out of the 40 *C. butyricum* strains tested, all were susceptible to vancomycin (MICs ≤ 4 mg/L), imipenem (MICs ≤ 2 mg/L), tigecycline (MICs ≤ 4 mg/L), linezolid (MICs < 4 mg/L), metronidazole (MICs ≤ 4 mg/L) and cefoxitin (MICs < 32 mg/L) (Table 2); strains were susceptible (MICs ≤ 1 mg/L) or intermediate (1 mg/L < MICs < 4 mg/L) to ofloxacin (Table 2). Although chloramphenicol resistance has been reported for *C. butyricum* reference strains [17], in this study all the strains were susceptible (MICs ≤ 8 mg/L). Antibiotic resistance was observed to penicillin G (MICs > 8 mg/L), amoxicillin (MICs > 8 mg/L) and piperacillin (MICs > 16 mg/L) for six strains, to tetracycline for three strains (MICs > 8 mg/L), to clindamycin for all strains (MICs ≥ 8 mg/L) and to erythromycin for two strains (MICs = 256 mg/L) (Table 2).

3.2. β-Lactam resistance

Most clostridia are susceptible to β-lactam agents. However, some species, such as *Clostridium difficile*, are less susceptible [15]. β-Lactamase production has been reported in only three species, namely *C. butyricum*, *Clostridium clostridioforme* and *Clostridium ramosum* [18], with no data on its incidence owing to the low number of strains tested. In this study, resistance to penicillin G (MICs > 8 mg/L), amoxicillin (MICs > 8 mg/L) and piperacillin (MICs > 16 mg/L) was observed for six strains (15%). Based on the nitrocefin

assay, all of the penicillin-resistant strains showed β-lactamase activity. In addition, the β-lactamases were inhibited by clavulanic acid, which has been reported to be a particularity of *C. butyricum* [19].

3.3. Tetracycline resistance

In this study, three *C. butyricum* strains showed resistance to tetracycline (MICs > 8 mg/L). One of the mechanisms involved in tetracycline resistance among anaerobic bacteria is ribosomal protection. Among clostridia, *tet*(M) was the most frequently identified tetracycline resistance gene for *C. difficile* [20,21] and *C. perfringens* [22]; the *tet*(O) [21] and *tet*(W) [23] genes were less frequently reported. In this study, the genomic DNA of all strains was used as a template for PCR amplification of the *tet*(M), *tet*(W), *tet*(O) and *tet*(Q) genes. Using the degenerated primers tet1 and tet2, PCR results showed that the *tet*(M), *tet*(W) and *tet*(Q) genes were absent in all of the isolates tested. Of the three tetracycline-resistant strains (strains 2, 22 and 33), only strain 22 showed amplification of the expected 1250-bp PCR product. The same 1250-bp PCR product was amplified for two non-resistant strains (strains 51 and 52). The nucleotide sequence of the 1250-bp fragment of strain 22 shared 71% identity with the mosaic *tet*(O/32/O) gene (accession no. AJ295238) from Clostridiaceae bacterium K10. Meanwhile, the nucleotide sequences of the same fragment for strains 51 and 52 showed 99% identity with *tet*(O) genes from *Enterococcus faecalis* (accession no. AY660532) and *Campylobacter jejuni* (accession no. M18896). To amplify the full-length genes of strains 22, 51 and 52, the primer combination OFF2/OFR3 was used (Table 1). This approach allowed the amplification of a 2000-bp PCR product with only the OFF2/OFR3 primers for strains 51 and 52 but not for strain 22. After sequencing, the 2000-bp PCR product nucleotide sequences from strains 51 and 52 shared 99% identity with the already mentioned *tet*(O)

genes. Attempts to amplify the full-length gene for strain 22 using multiple primer combinations were unsuccessful (data not shown).

To investigate the tetracycline resistance mechanism of strains 2 and 33, all strains were screened for the *tet*(P), *tet*(K) and *tet*(L) tetracycline efflux pumps genes. Indeed, such genes have been identified in *C. difficile* [24] and *C. perfringens* [25]. PCR screening showed that *tet*(K) and *tet*(L) were absent from all the strains tested. The *tet*(P) gene (accession no. EDT76835.1), which was found to be present on the *C. butyricum* 5521 sequenced genome (accession no. NZ ABDT00000000), was identified for all strains except strains 22, 51 and 52. RT-PCR experiments confirmed *tet*(P) expression for tetracycline-resistant strains 2 and 33. However, *tet*(P) expression was also observed for all susceptible strains. The fact that *tet*(P) may be tightly regulated at the transcriptional or translational levels may explain these observations [26].

3.4. Erythromycin and clindamycin resistance

Macrolide–lincosamide–streptogramin B (MLS_B) resistance in clostridia is mostly encoded by rRNA methyltransferase *erm* genes. In *C. difficile* and *C. perfringens* it involves the *erm*(B), *erm*(Q) or *erm*(F) genes [21,25]. With regard to *C. butyricum*, one clinical isolate was reported to carry *erm*(B) and *erm*(F) genes [27]. In this study, all strains showed MICs $\geq$ 8 mg/L to clindamycin and two were resistant to erythromycin (MICs of 256 mg/L). PCR amplification for *erm* genes showed the absence of *erm*(B), *erm*(Q) and *erm*(F) as an explanation for the observed resistance (data not shown). Such observations have been reported for other *erm*-negative clostridia with erythromycin and clindamycin resistance [28]. These results suggest the presence of an alternative resistance mechanism(s). Interestingly, an *lmrB* efflux gene homologue (accession number no. EDT76011) was

found to be present on the *C. butyricum* 5521 sequenced genome (accession no. NZ ABDT00000000). Although efflux pumps may participate in clindamycin resistance in anaerobic bacteria [29], it was not reported for clostridia. In this study, PCR amplification of the chromosomal *C. butyricum* efflux gene homologue *lmrB* was positive for 70% of the strains. RT-PCR experiments showed that 75% of the *lmrB*-positive strains showed gene expression. This suggests the involvement of other mechanism(s) in the observed resistance.

4. Conclusion

This study provides recent information on the status of *C. butyricum* antimicrobial susceptibility and shows that this human gut commensal bacterium is susceptible to common anti-anaerobe antimicrobial agents. The prevalence of this species in preterm neonates may be not linked to a high degree of resistance to antimicrobial agents. This study provides new data on the genetic antibiotic resistance determinants of *C. butyricum* and reports for the first time the acquired resistance to tetracycline by ribosomal protection genes *tet*(O) and a potential mosaic *tet*(O/32/O) homologue.

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Competing interests

None declared.

Ethical approval

Not required.

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Table 1

Primers used for PCR and RT-PCR experiments

Genes	Primers	Primer sequences (5'→3')
tet(W), *tet*(M), *tet*(Q)	tet1	GCTCAYGTTGAYGCAGGAA
	tet2	AGGATTTGGCGGSACTTCKA
tet(O)	OFF2	TTGTTTTGGGGCTATTGGAG
	OFR3	TATATGACTTTTGCAAGCTG
tet(P)	TetPCbutF	TTCTTGCTCATGTTGATGCC
	TetPCbutR	GAAGTATACTCAATATCAGC
	TetP-ShrtF	GGCCCTGTTTCAACATTCAT
	TetP-ShrtR	ATCCACTTCCATGGGAACAA
tet(K)	TetKF	GTACAAGGAGTAGGATCTGCTGCAT
	TetKR	TTATTCCCCCTATTGAAGGACCTAA
tet(L)	TetLF	TGAACGTCTCATTACCTGATATTGC
	TetLR	TTTGGAATATAGCGAGCAAC
erm(B)	ErmBV	AATAAGTAAACAGGTTACGT
	ErmBR	CTACTGACAGCTTCCAAGGAGC
	ErmBE5	CTCAAAACTTTTTAACGAGTG
	ErmBE6	CCTCCCGTTAAATAATAGATA
erm*(F)*	ErmF1	CGGGTCAGCACTTTACATTTG
	ErmF2	GGACCTACCTCATAGCAAG
	ErmFS3	GAGAGGAAAGAGAGACAATGTC
	ErmFS4	TTTATCTACTCCGATAGCTTCC
erm(Q)	ErmQ3	GGAGGAAATAAAATGATTATGAATGG
	ErmQ4	CACATAAAGCTTCTGTTATATGACC
lmrB	LmrBF	GTTTTAGTACCAGTTACAGC
	LmrBR	CCAGAAGCAACTGCACTCCA
	LmrB-ShrtF	GCTTTAACTCCGGTAGCTGGT
	LmrB-ShrtR	AGCCACTGTCTGTGATGGTG
recA	RecA-F	GCAGAGCATGCATTAGATCCT
	RecA-R	GAATCTCCCATTTCCCCTTC

PCR, polymerase chain reaction; RT-PCR, reverse transcription PCR.

Table 2

Susceptibility of *Clostridium butyricum* isolates to antimicrobials agents

Antimicrobial agent	No. of strains at an MIC (mg/L) of:													MIC (mg/L)	
	0.0625	0.125	0.250	0.5	1	2	4	8	16	32	64	128	256	MIC_{50}	MIC_{90}
Penicillin G			7	12	8	7					6			1	64
Amoxicillin		8	18	8					6					0.125	16
Piperacillin				5	16	11	2				6			1	64
Cefoxitin							12	18	10					8	16
Imipenem			9	13	17	1								0.5	1
Vancomycin				16	24									1	1
Tetracycline	27	3					1	6	2	1				0.0625	8
Tigecycline	30	3	5	1	1									0.0625	0.250
Erythromycin				34	4								2	0.5	1
Clindamycin								7	30	2		1		16	16
Ofloxacin		1		2	33	4								1	1
Metronidazole		6	23	11										0.125	0.5
Chloramphenicol				5	32	3								1	1
Linezolid				2	34	3	1							1	1

MIC, minimal inhibitory concentration; $MIC_{50/90}$, MICs for 50% and 90% of the organisms, respectively.