CLINICAL SCIENCE

Antimicrobial resistance and prevalence of resistance genes in intestinal *Bacteroidales* strains

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OBJECTIVE: This study examined the antimicrobial resistance profile and the prevalence of resistance genes in *Bacteroides* spp. and *Parabacteroides distasonis* strains isolated from children's intestinal microbiota.

METHODS: The susceptibility of these bacteria to 10 antimicrobials was determined using an agar dilution method. β-lactamase activity was assessed by hydrolysis of the chromogenic cephalosporin of 114 *Bacteriodales* strains isolated from the fecal samples of 39 children, and the presence of resistance genes was tested using a PCR assay.

RESULTS: All strains were susceptible to imipenem and metronidazole. The following resistance rates were observed: amoxicillin (93%), amoxicillin/clavulanic acid (47.3%), ampicillin (96.4%), cephalexin (99%), cefoxitin (23%), penicillin (99%), clindamycin (34.2%) and tetracycline (53.5%). β-lactamase production was verified in 92% of the evaluated strains. The presence of the *cfiA, cepA, ermF, tetQ* and *nim* genes was observed in 62.3%, 76.3%, 27%, 79.8% and 7.8% of the strains, respectively.

CONCLUSIONS: Our results indicate an increase in the resistance to several antibiotics in intestinal *Bacteroides* spp. and *Parabacteroides distasonis* and demonstrate that these microorganisms harbor antimicrobial resistance genes that may be transferred to other susceptible intestinal strains.

KEYWORDS: *Bacteroides* spp.; *Parabacteroides distasonis*; β-lactamase activity; Antimicrobial resistance; Resistance genes.

Nakano V, Nascimento e Silva A, Merino VRC, Wexler HM, Avila-Campos MJ. Antimicrobial resistance and prevalence of resistance genes in intestinal *Bacteroidales* strains. Clinics. 2011;66(4):543-547.

Received for publication on September 11, 2010; **First review completed on** October 18, 2010; **Accepted for publication on** December 17, 2010

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INTRODUCTION

Bacteroides and *Parabacteroides* species are components of the colon resident microbiota, and both genera belong to the order *Bacteroidales*.[1] Species of these genera are often associated with opportunistic mixed infections, such as intra-abdominal, obstetric-gynecologic and diabetic foot infections. In addition, these microorganisms are able to develop resistance to several antimicrobial drugs.[2] Although antibiotics with good activity against these bacteria are currently available, high frequencies of resistance to some antimicrobials have been reported in several countries.[2,3]

Bacteroides and *Parabacteroides* species produce endogenous β-lactamases, the most important mechanism of resistance to β-lactam antibiotics. *Bacteroides fragilis* is the most frequently isolated bacteria from infectious diseases, and it exhibits high levels of resistance to β-lactam drugs[4] compared with other *Bacteroidales* because of the production of cephalosporinases and penicillinases encoded by the *cepA* gene.[5]

Bacterial resistance to imipenem, ertapenem and meropenem arises because of the production of metallo-β-lactamase (class B) encoded by the *cfiA* gene, but this resistance is rarely observed in *Bacteroides* and *Parabacteroides* species.[6] Strains harboring ''silent'' *cepA* or *cfiA* genes appear to be resistant to penicillin, cephalosporin or carbapenem. Conversely, some *B. fragilis* strains harboring either *cepA* or *cfiA* genes are susceptible to β-lactams, but after antibiotic pressure, they become resistant because of an insertion sequence (IS) in the upstream region of these genes.[7]

Clindamycin resistance rates have been shown to vary from 10% to 42% in intestinal *Bacteroidales* strains worldwide.[3,8] Clindamycin resistance is encoded by the *ermF* gene, which confers resistance to macrolides, lincosamides and streptogramin B via a 23S rRNA mechanism, which produces the methylases ErmF, EmFS, ErmG and ErmB.[9]

Metronidazole resistance in anaerobic bacteria appears to be associated with the *nim A* to *G* genes that are transcribed by promoters located in different IS-producing nitroimidazole reductases, which transform 4- or 5-nitroimidazole

genes to 4- or 5-aminoimidazoles.[10] Nitroimidazole resistance in *Bacteroides* spp. and *Parabacteroides distasonis* is not commonly observed. Although non-*nim* genes associated with imidazole resistance have been reported, this resistance might be due to an extensive use of metronidazole. However, the exact mechanism of this resistance remains undefined.[11]

The aim of this study was to determine the antimicrobial resistance profile and the prevalence of resistance genes in *Bacteroides* spp. and *Parabacteroides distasonis* strains isolated from children's intestinal microbiota.

METHODS AND MATERIALS

Bacteria

A total of 114 intestinal *Bacteroidales* samples (66 *Bacteroides fragilis*; 14 *B. vulgatus*; 7 *B. uniformis;* 7 *B. ovatus;* 2 *B. eggerthii*; 2 *B. thetaiotaomicron* and 16 *Parabacteroides distasonis*) isolated from 39 fecal samples from children were evaluated. Children from 2 children's hospitals and 2 day care centers (São Paulo, SP, Brazil) were selected for this study, with ages ranging from 2 months to 8 years old. None of the subjects received antibiotic therapy prior to sample collection. Fecal samples were collected from April to December 2000. Stools were plated onto *Bacteroides fragilis*-bile-esculin agar and identified using an established methodology[12]. The strains were stored at -80℃ in 10% skim milk. This study was approved by the Ethics Commission of the Instituto de Ciências Biomédicas, USP (158/CEP).

Susceptibility Testing

Antimicrobial susceptibility tests were performed using an agar dilution method in Wilkins & Chalgren agar in accordance with the recommendations of the Clinical and Laboratory Standards Institute (CLSI).[13] The antibiotics used were as follows: amoxicillin, ampicillin, cephalexin, clindamycin and tetracycline (Luper Ind. Farm. Ltd., SP, Brazil), cefoxitin and imipenem (Merck Sharp & Dohme, SP), amoxicillin/clavulanic acid (Smithkline Beechman Brazil Ltd., SP), metronidazole (Aventis Farm. Ltd., SP) and penicillin (Prodoti Lab. Farm. Ltd., SP). Briefly, media containing twofold serial dilutions of antimicrobial agents ranging from 0.25 to 512 μg/ml were inoculated with 1.5×10^5 cfu delivered by a Steers replicator. Media without antibiotics were used as controls. Plates were incubated in anaerobic conditions (90% N_2/10% CO_2) at 37℃ for 48 h. The Minimal Inhibitory Concentration (MIC) was defined as the lowest concentration of each antimicrobial agent able to inhibit visible bacterial growth. All tests were performed in duplicate. The *B. fragilis* ATCC 43858 was included as a control in all assays to assess the reliability of the methods.

Determination of β-lactamase activity

Hydrolysis of the chromogenic cephalosporin (Nitrocefin, Oxoid Ltd., São Paulo, SP, Brazil) was used to observe enzyme production. β-lactamase activity was expressed semi-quantitatively; negative β-lactamase activity was indicated by a yellow color and positive by a red color. The penicillin-resistant and β-lactamase-positive strain *B. fragilis* ATCC 43858 was used as a control.

Detection of resistance genes by a PCR assay

Bacterial genomic DNA was obtained using an Easy-DNA kit (Invitrogen do Brasil Ltd., São Paulo, SP, Brazil) according to the manufacturer's instructions. PCR assays were used to detect the presence of resistance genes (cfiA, *cepA, ermF, tetQ* and *nim*) and the insertion sequences (*IS942* and *IS1186*) associated with *cfiA* gene expression.[5] DNA amplifications were performed in volumes of 25 μL containing 1 X PCR buffer (Invitrogen), 2.5 mM $MgCl_2$, 0.2 mM dNTP mix (Invitrogen), 0.4 μM of each primer (Invitrogen), 0.5 U of Platinum *Taq* DNA polymerase (Invitrogen and 10 ng of DNA. Amplifications were performed in a thermal cycler (PerkinElmer Amp PCR System 9700). Table 1 shows the PCR conditions, including the genes, primer sequences and cycles. Amplification products were analyzed by electrophoresis in a 1% agarose gel (in 1X TBE buffer), stained with ethidium bromide and photographed under UV light.

Statistical Analysis

All statistical analyses were performed using GraphPad InStat statistical analysis software (version 3.05, GraphPad Software) with a one-way ANOVA. A difference of $p\leq0.05$ was considered statistically significant.

RESULTS

All tested strains were susceptible to imipenem and metronidazole. Cefoxitin was active against 77% of the tested bacteria. In addition, clindamycin was active against 65.8% of the tested bacteria, combined amoxicillin/clavulanic acid against 52.7%, and tetracycline against 46.4%. All intestinal *Bacteriodales* species exhibited antimicrobial resistance ranging from 23% to 99% (Table 2). Most strains (92%) were able to produce β-lactamases (Table 3). All strains harbored at least one of the resistance genes evaluated. For

Table 1 - Resistance genes, oligonucleotide sequences and PCR conditions used to detect target genes

Resistanc Genes	Oligonucleotide Sequence 5′→3′	Amplification Cycles	Reference
cepA	TTT CTG CTA TGT CCT GCC C ATC TTT CAC GAA GAC GGC	35 cycles: 94℃×60 sec 52℃×60 sec 72℃×60 sec	5
cfiA	ATG GTA CCT TCC AAC GGG CAC GAT ATT GTC GGT CGC	35 cycles: 94℃×60 sec 56℃×60 sec 72℃×60 sec	5
IS1186	TGA CCT ACA ACA TCT TCC G GGT TGT TGA TAA CAA TCA TCC C	35 cycles: 94℃×60 sec 50℃×60 sec 72℃×2 min	5
IS942	TCC TCA ATA CAT GAG CCG C GGT TGT TGA TAA CAA TCA TCC C	35 cycles: 94℃×60 sec 50℃×60 sec 72℃×2 min	5
tetQ	ACT TCC GTA ACC GAG AAT CTG CTG TAC CGG ATA GAC TTT GGC TTT TGC	40 cycles: 94℃×60 sec 50℃×60 sec 72℃×40 sec	30
ermF	CGG GTC AGC ACT TTA CTA TTG GGA CCT ACC TCA TAG ACA AG	35 cycles: 94℃×30 sec 50℃×30 sec 72℃×2 min	23
nim	ATG TTC AGA GAA ATG GGG CGT AAG CG GCT TCC TTG CCT GTC ATG TGC TC	34 cycles: 94℃×60 sec 55℃×60 sec 72℃×30 sec	31

Table 2 - Resistance profiles of intestinal *Bacteroidales* species to 8 antibiotics.

Antibiotics*	% resistance**							
	B. fragilis (n = 66)	*B. vulgatus* (n = 14)	*B. uniformis* (n = 7)	*B. ovatus* (n = 7)	*B. eggerthii* (n = 2)	*B. thetaiotaomicron* (n = 2)	*P. distasonis* (n = 16)	*Bacteroides* spp. and *Parabacteroides* sp. (n = 114)
Amoxicillin	92.4	85.7	100	100	100	100	93.7	93
Amoxicillin/ Clavulanic acid	40.9	42.8	0	85.7	100	100	68.7	47.3
Ampicillin	98.4	92.8	100	100	100	100	87.5	96.4
Cephalexin	100	92.8	100	100	100	100	100	99
Cefoxitin	7.5	0	14.2	0	0	0	12.5	23
Clindamycin	31.8	100	0	71.4	0	0	43.7	34.2
Penicillin	100	92.8	100	100	100	100	100	99
Tetracycline	59	50	28.5	71.4	100	0	43.7	53.5

*Breakpoints used in accordance with CLSI (2007): Amoxicillin (8 μg/mL); Amoxicillin/clavulanic acid (8 μg/mL); Ampicillin (1 μg/mL); Cephalexin (8 μg/mL); Cefoxitin (32 μg/mL); Clindamycin (4 μg/mL); Imipenem (8 μg/mL); Metronidazole (16 μg/mL); Penicillin (1 μg/mL) and Tetracycline (8 μg/mL).
**All strains were susceptible to imipenem and metronidazole.
***B. fragilis* ATCC 43858 was resistant to amoxicillin, ampicillin, cephalexin, clindamycin and penicillin.

example, 71 strains (62.3%) harbored the *cfiA* gene and were susceptible to imipenem. Moreover, mobile elements were observed in 2 *B. fragilis* (*IS1186* and *IS942*) strains, 1 *B. vulgatus* (*IS1186*) strain and 1 *P. distasonis* (*IS942*) strain, but none carried the *cfiA* gene. The *cepA* gene was present in 87 (76.3%) of the tested *Bacteroidales* species, and high resistance values to some antimicrobials, including cephalexin and penicillin (99%), ampicillin (96.4%), and amoxicillin (93%), were observed, suggesting the possibility of an association between the presence of these genes and the resistance to cephalosporin and penicillin. Out of 39 clindamycin-resistant strains, 31 (79.5%) harbored the *ermF* gene. While 91 (79.8%) of the tested strains harbored the *tetQ* gene, only 61 (67%) were resistant to tetracycline. Bacterial strains were susceptible to metronidazole. However, 9 strains (5 *B. fragilis*, 1 *B. vulgatus*, 2 *B. uniformis* and 1 *P. distasonis*) harbored the *nim* gene. The presence of resistance genes in all tested *Bacteroides* and *Parabacteroides* strains was statistically significant ($p<0.001$), and $p<0.01$ was observed in *B. fragilis* strains. Table 3 shows the distribution of the resistance genes in *Bacteroides* spp. and *P. distasonis*.

DISCUSSION

Bacteroidales species are important anaerobe components of the resident intestinal microbiota, and they are potential endogenous pathogens. *Bacteroides* species and *P. distasonis* have been shown to induce different infections in humans.[2] These intestinal anaerobes are resistant to several penicillins and cephalosporins,[14] but the exact mechanism of this resistance is unknown.

In this study, a high rate of β-lactamase-producing strains (92%) was observed in accordance with previous studies.[3,15] In addition, some resistant strains can produce β-lactamases that are encoded by plasmid-borne or chromosomal *cepA* genes, and these enzymes are responsible for the increase in antibiotic resistance.

Most anaerobic bacteria are susceptible to imipenem,[4,17] although high rates of resistance to this drug have been reported.[2,14] Moreover, in *Bacteroidales* the *cfiA* gene has been detected at a low rate.[16,18,19] In this study, 71 (62.3%) of the tested strains harbored the *cfiA* gene, but no imipenem-resistant strains were observed. Conversely, high detection rates of the *cfiA* gene suggest that these strains act as reservoirs for antibiotic resistance genes, which is in accordance with the results of Garcia et al.[20] The role of the *cfiA* gene in these intestinal strains remains unclear, and further studies are necessary to understand its presence. Moreover, *cfiA*-positive *Bacteroidales* strains did not harbor either *IS942* or *IS1186* elements, which has also been demonstrated by Soki et al.[18] and Walsh et al.[21] In addition, strains susceptible to imipenem did not harbor the IS promoter.

The production of cephalosporinases and penicillinases encoded by the *cepA* gene is commonly observed in *Bacteroides* spp. and *Parabacteroides distasonis*.[5] In this study, 87 (82.8%) of 105 β-lactamase-producing strains harbored

Table 3 - Distribution of resistance genes and β-lactamase production in intestinal *Bacteroides* spp. and *P. distasonis.*

Species (n)	Genes					β-lactamase production
	cfiA	*cepA*	*ermF*	*tetQ*	*nim*	
	n° (%)	n° (%)	n° (%)	n° (%)	n° (%)	n° (%)
B. fragilis (66)	51 (77.2)	53 (80.3)	16 (24.2)	54 (81.8)	5 (7.5)	60 (90.9)
B. vulgatus (14)	5 (35.7)	11 (78.5)	5 (35.7)	7 (50)	1 (7.14)	13 (92.8)
B. uniformis (7)	6 (85.7)	7 (100)	0 (0)	5 (71.4)	2 (28.5)	7 (100)
B. ovatus (7)	1 (14.2)	1 (14.2)	1 (14.2)	6 (85.7)	0 (0)	7 (100)
B. eggerthii (2)	0 (0)	2 (100)	1 (50)	2 (100)	0 (0)	2 (100)
B. thetaiotaomicron (2)	2 (100)	2 (100)	2 (100)	2 (100)	0 (0)	1 (50)
P. distasonis (16)	6 (37.5)	11 (68.7)	6 (37.5)	15 (93.7)	1 (6.25)	15 (93.7)
TOTAL (114)	**71 (62.3)**	**87 (76.3)**	**31 (27)**	**91 (79.8)**	**9 (7.8)**	**105 (92)**

the *cepA* gene, suggesting that some strains were able to produce this enzyme using mechanisms other than the cepA gene.

Most β-lactamase-producing strains were susceptible to cefoxitin, with a resistance rate of only 23% (Table 2). These data are supported by previously published studies.[3,8] Moreover, the combination of amoxicillin and clavulanic acid did not show good activity against 47.3% of the tested strains, in accordance with Wybo et al.[3] and Roberts et al.[17]

Clindamycin is a semi-synthetic drug used extensively in the treatment of anaerobic infections.[22] However, bacterial resistance to this drug has significantly increased over the last two decades. In this study, 34.2% of strains were observed to be clindamycin resistant, in accordance with Betriu et al.[14] Intestinal *Bacteroidales* strain resistance rates to clindamycin have been shown to vary between countries from 39% to 41%.[3,4,16]

The *ermB*, *ermF*, *ermG*, and *ermS* genes are the most common determinants of genetic resistance in intestinal *Bacteroidales* strains.[23] Of the 39 (34.2%) clindamycin-resistant strains in this study, only 10 harbored the *ermF* gene. Bacterial resistance to clindamycin can arise because of the presence of the *ermB* or *ermG* genes[24] or by other mechanisms, such as efflux pumps.[25] In addition, clindamycin resistance among *Bacteroidales* species has increased in several countries.[26,27] This alarming resistance to clindamycin among *Bacteroides* spp. and *P. distasonis* makes its use unacceptable for the empiric therapy of severe anaerobic infections.

Tetracycline is one of the most widely used antibiotics worldwide, but its use has decreased because of the high resistance rates observed in various microorganisms, including *Bacteroides* spp. and *Parabacteroides distasonis*.[16] Efflux pumps, ribosome protection and tetracycline modification are the main mechanisms of bacterial resistance to tetracycline. However, ribosome protection appears to be the most widespread in nature.[28] The *tetQ* and *tetM* genes encoding the ribosome-protecting proteins are often associated with conjugative transposons.[29] In this study, 53.5% of the tested strains were resistant to tetracycline, and among the 91 (79.8%) of 114 total strains harboring the *tetQ* gene, 61 (67%) showed resistance to tetracycline. This result suggests that these bacteria may become resistant either by activating other genes, such as *tetM*, *tetK*, *tetL* and *tetO*, or by another mechanism of resistance that remains to be clarified.

All tested strains were susceptible to metronidazole in accordance with Odou et al.[4] However, other studies have noted an increase in the rate of resistance to metronidazole.[3,11] In this study, only 9 (7.8%) strains harbored the *nim* gene. Metronidazole resistance associated with *nim* has been described in *Bacteroides* spp. and *Parabacteroides distasonis* strains from different geographic regions.[11] However, resistance to metronidazole does not depend on the presence of *nim* genes, and the true role of these genes is not yet clear. In addition, *nim*-negative strains expressing high levels of resistance to metronidazole have been sporadically isolated, suggesting an additional mechanism of resistance[11] and also justifying additional studies concerning the susceptibility profile and detection of *nim* genes.

Few studies have addressed antimicrobial susceptibility profiles and the detection of resistance genes in intestinal anaerobic resident microbiota in Brazil, especially with a focus on children. Careful monitoring of antimicrobial resistance and detection of these genes might be of interest, verifying the presence and spread of intestinal *Bacteriodales* strains with resistance markers to different antimicrobials in different countries.

ACKNOWLEDGEMENTS

The authors thank Mrs. Zulmira Alves de Souza for her technical support. This study was supported by Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP Grant 08/57330-4 and 09/03792-0).

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