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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 Bacteroidales 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%), cephalaxin (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.

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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 drugs⁴ 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.\textsuperscript{10} Nitroimidazole resistance in \textit{Bacteroides} spp. and \textit{Parabacteroides distasonis} is not commonly observed. Although non-nim genes associated with nitroimidazole resistance have been reported, this resistance might be due to an extensive use of metronidazole. However, the exact mechanism of this resistance remains undefined.\textsuperscript{11}

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

### METHODS AND MATERIALS

#### Bacteria

A total of 114 intestinal \textit{Bacteroidales} samples (66 \textit{Bacteroides fragilis}; 14 \textit{B. vulgatus}; 7 \textit{B. uniformis}; 7 \textit{B. ovatus}; 2 \textit{B. eggerthii}; 2 \textit{B. thetaiotaomicron} and 16 \textit{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 \textit{Bacteroides fragilis}-bile-esculin agar and identified using an established methodology.\textsuperscript{12} The strains were stored at -80°C 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).\textsuperscript{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 (Prodotti Lab. Farm. Ltd., SP). Briefly, media containing twofold serial dilutions of antimicrobial agents ranging from 0.25 to 512 \textmu g/ml were inoculated with 1.5×10\textsuperscript{6} cfu delivered by a Steers replicator. Media without antibiotics were used as controls. Plates were incubated in anaerobic conditions (90% N\textsubscript{2}/10% CO\textsubscript{2}) at 37°C 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 \textit{B. fragilis} ATCC 43858 was included as a control in all assays to assess the reliability of the methods.

#### Determination of \textbeta-lactamase activity

Hydrolysis of the chromogenic cephalosporin (Nitrocefin, Oxoid Ltd., São Paulo, SP, Brazil) was used to observe enzyme production. \textbeta-lactamase activity was expressed semi-quantitatively; negative \textbeta-lactamase activity was indicated by a yellow color and positive by a red color. The penicillin-resistant and \textbeta-lactamase-positive strain \textit{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 (\textit{cepA}, \textit{ermF}, \textit{tetQ} and \textit{nim}) and the insertion sequences (\textit{IS942} and \textit{IS1186}) associated with \textit{cepA} gene expression.\textsuperscript{9} DNA amplifications were performed in volumes of 25 \textmu l containing 1 X PCR buffer (Invitrogen), 2.5 mM MgCl\textsubscript{2}, 0.2 mM dNTP mix (Invitrogen), 0.4 \mu M 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 \textit{p}≤0.05 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 \textit{Bacteroidales} species exhibited antimicrobial resistance ranging from 23% to 99% (Table 2). Most strains (92%) were able to produce \textbeta-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 |
|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|
| Resistant Genes                              | Oligonucleotide Sequence 5’–3’                  | Amplification Cycles                          | Reference                                    |
| \textit{cepA}                                 | TTT CTG CTA TGT CCT GCC C                        | 35 cycles:                                    | 5                                            |
|                                                | ATC TTT CAC GAA GAC GGC                          |                                              |                                              |
| \textit{cfIA}                                 | ATG GTA CTA TCC AAC GGG                          | 35 cycles:                                    | 5                                            |
|                                                | CAG GAT ATT GTC GGT CGC                          |                                              |                                              |
| \textit{IS1186}                               | TGA CCT ACA ACA TCT TCC G                        | 35 cycles:                                    | 5                                            |
|                                                | GGT TGT TGA TAA CAA TCA TCC C                    |                                              |                                              |
| \textit{IS942}                                | TCC TCA ATA CAT GAG CCG C                        | 35 cycles:                                    | 5                                            |
|                                                | GGT TCC TGA TAA CAA TCA TCC C                    |                                              |                                              |
| \textit{tetQ}                                 | ACT TCC GTA ACC GAG AAT                          | 40 cycles:                                    | 30                                           |
|                                                | CTG CTG                                       |                                              |                                              |
| \textit{ermF}                                 | TAC CGG ATA GAC TTT GCC                         | 35 cycles:                                    | 23                                           |
|                                                | TTT TGC                                       |                                              |                                              |
| \textit{nim}                                  | ATG TCC AGA GAA ATG GGG                         | 34 cycles:                                    | 31                                           |
|                                                | CGT AAG CG                                     |                                              |                                              |
|                                                | GCT TCC TTG CCT GTC ATG                        | 55°C:60 sec                                    |                                              |
|                                                | TGC TC                                        |                                              |                                              |
|                                                | 72°C:30 sec                                    |                                              |                                              |
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 cepA gene. The cepA gene was present in 87 (76.3%) of the tested Bacteroidales species, and high resistance values to some antimicrobials, including cepalexin 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. These intestinal anaerobes are resistant to several penicillins and cephalosporins, 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. 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 aerobic bacteria are susceptible to imipenem, although high rates of resistance to this drug have been reported. Moreover, in Bacteroidales the cepA gene has been detected at a low rate. In this study, 71 (62.3%) of the tested strains harbored the cepA gene, but no imipenem-resistant strains were observed. Conversely, high detection rates of the cepA gene suggest that these strains act as reservoirs for antibiotic resistance genes, which is in accordance with the results of Garcia et al. The role of the cepA gene in these intestinal strains remains unclear, and further studies are necessary to understand its presence. Moreover, cepA-positive Bacteroidales strains did not harbor either IS942 or IS1186 elements, which has also been demonstrated by Soki et al. and Walsh et al. 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. In this study, 87 (82.8%) of 105 β-lactamase-producing strains harbored

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

| Antibiotics | 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)           | 100 (100)         | 100 (100)            | 93.7 (90.9)                | 93 (90.9)               | 96.4 (92.8)                      |
| Amoxicillin/Clavulanic acid | 40.9 (42.8) | 0 (100)             | 85.7 (100)          | 100 (100)         | 100 (100)            | 68.7 (94.7)                | 47.3 (87.5)             | 99 (92.8)                        |
| Ampicillin  | 98.4 (92.8)         | 100 (100)           | 100 (100)           | 100 (100)         | 100 (100)            | 100 (100)                  | 100 (100)               | 100 (100)                       |
| Cefoxitin   | 7.5                 | 14.2                | 0 (0)               | 0 (0)             | 0 (0)                | 12.5 (12.5)                | 23 (23)                 | 34.2 (34.2)                     |
| Clindamycin | 31.8                | 0 (0)               | 71.4 (100)          | 0 (0)             | 0 (0)                | 43.7 (43.7)                | 53.5 (53.5)            | 3.8 (3.8)                       |
| Penicillin  | 100 (100)           | 100 (100)           | 100 (100)           | 100 (100)         | 100 (100)            | 100 (100)                  | 100 (100)               | 99 (92.8)                       |
| Tetracycline| 59 (50)             | 28.5                | 71.4 (100)          | 100 (100)         | 0 (0)                | 43.7 (53.5)                | 100 (100)              | 99 (92.8)                       |

*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).

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

| Species (n) | cfiA n (%) | cepA n (%) | ermA n (%) | tetQ n (%) | nim n (%) | β-lactamase production n (%) |
|-------------|------------|------------|------------|------------|-----------|-------------------------------|
| B. fragilis (66) | 51 (77.2) | 53 (80.3) | 16 (24.2) | 54 (81.8) | 5 (7.5) | 54 (81.8)                       |
| B. vulgatus (14) | 5 (35.7)  | 11 (78.5) | 5 (35.7)  | 7 (50)     | 1 (7.14) | 7 (50)                         |
| B. uniformis (7) | 8 (85.7)  | 7 (100)    | 0 (0)      | 5 (71.4)   | 2 (28.5) | 7 (100)                        |
| B. ovatus (7)  | 1 (12.5)  | 1 (12.5)   | 1 (12.5)   | 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 (100) |
| 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 (78.8) | 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. 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. and Roberts et al.

Clindamycin is a semi-synthetic drug used extensively in the treatment of anaerobic infections. 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. Intestinal Bacteroidales strain resistance rates to clindamycin have been shown to vary between countries from 39% to 41%. The ermB, ermF, ermG, and ermS genes are the most common determinants of genetic resistance in intestinal Bacteroidales strains. 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 or by other mechanisms, such as efflux pumps. In addition, clindamycin resistance among Bacteroidales species has increased in several countries. 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. 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. The tetQ and tetM genes encoding the ribosome-protecting proteins are often associated with conjugative transposons. 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. However, other studies have noted an increase in the rate of resistance to metronidazole. 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. 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 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 Bacteroidales strains with resistance markers to different antimicrobials in different countries.

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