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Plasmid-Related Resistance to Cefoxitin in Species of the *Bacteroides fragilis* Group Isolated from Intestinal Tracts of Calves

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Abstract. Species of the *Bacteroides fragilis* group are considered the most common anaerobe in human and animal infections and also harbor plasmids conferring resistance to several antibiotics. In this study, resistance to cefoxitin, plasmid profile and β-lactamase production in species of the *B. fragilis* group isolated from intestinal tracts of calves were evaluated. One hundred sixty-one *B. fragilis* group bacteria isolated from calves with and without diarrhea were analyzed. Cefoxitin susceptibility was performed using an agar dilution method, β-lactamase production by using a nitrocefin method, and plasmid extraction by using a commercial kit. Minimal inhibitory concentration values for cefoxitin ranged from 32 to > 512 μg/ml, and 47 bacteria (29.2%) were resistant to cefoxitin (breakpoint 16 μl). Only seven isolates harbored plasmids varying from 6.0 to 5.0 kb, and a 5.5-kb plasmid in *B. vulgatus* Bd26e and *B. fragilis* Bc5j might be related to cefoxitin resistance. β-lactamase was detected in 33 (70.2%) isolates. The *cepA* gene was observed in total DNA and in the 5.5-kb plasmid. The plasmid presence in organisms isolated from cattle may be important in ecologic terms, and it needs further study.

The *Bacteroides fragilis* group represents a significant part of the human intestinal microbiota, and these organisms are also of clinical importance as a cause of serious infections in man, such as brain, lung, intra-abdominal and intrapelvic abscesses, peritonitis, and sepsis [1]. Species of the *B. fragilis* group are part of the intestinal resident microbiota in animals, and they can produce several infectious processes in calves such as abscesses, septicemia, endocarditis, diarrhea, and urinary infection. The genus *Bacteroides* is predominant in these animals' colons, but other organisms, such as *Eubacterium, Bifidobacterium, Clostridium, Fusobacterium, Ruminococcus, Peptococcus*, and *Peptostreptococcus* have been found [2].

Members of the *B. fragilis* group are considered the most common anaerobe in human and animal infections and also harbor genetic elements that act in the transmission of resistance genes to antibiotics to different bacterial groups. Sóki et al. [3] demonstrated that 3.7-MDa plasmids are the most frequently observed in species of the *B. fragilis* group.

These bacteria have shown an increase of the resistance to most of traditionally antimicrobial agents used for treating anaerobic infections. The β-lactam antibiotic cefoxitin is a common drug used for the treatment of infections caused by *B. fragilis* [4]. However, β-lactamase production is the most important mechanism of resistance to β-lactam antibiotics in Gram-negative bacteria, especially in species of the *B. fragilis* group [5]. Two genotypically distinct β-lactam classes mediated for *cfiA* (encoding a metallo-β-lactamase of Ambler's class B) and *cepA* (encoding an endogenous cephalosporinase β-lactamase of Ambler's class A) have been described in *Bacteroides* [5, 6]. However, an enzyme encoded by *cfxA* gene that confers resistance to cefoxitin among strains of the *B. fragilis* group has been described, but its expression seems to be rare [7].

In Brazil, high levels of resistance to cefoxitin in species of the *B. fragilis* group isolated from humans has

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been observed as has the presence of the *cepA* and *cfiA* genes [7, 8]. Variation in resistance rates may be caused selective pressure of antimicrobial agents used in each geographic region [9, 10]. Moreover, the *cfiA* gene on plasmid or chromosome has also been observed, but no evidence showing an extrachromosomal location of the *cepA* gene has been reported [11]. Because cattle are a food source for humans, and the presence of organisms carrying resistance genes for antibiotics has been observed in the intestinal tracts of these animals, these genetic elements can be transferred interspecies or intraspecies.

This study is important because diarrhea in calves causes an economic loss in different countries and because these organisms can be transferred to humans as a cross-infection. Thus, in this study, cefoxitin resistance, plasmid profile, and β-lactamase production in species of the *B. fragilis* group isolated from the intestinal tracts of calves were evaluated.

Material and Methods

One hundred sixty-one bacteria of the *B. fragilis* group were isolated from 52 calves, 31 of which had acute diarrhea. Animals were female; ranged in age from 7 to 90 days old; and belonged to five farms from the Brazilian cities of São Paulo and Ribeirão Preto. Calves drank water ad libitum, and none of them had been treated with antibiotics. Bacteria were isolated onto *B. fragilis*–bile-esculin agar, incubated under anaerobic conditions (90% nitrogen and 10% carbon dioxide), and then identified using the RAPID 32A kit (bioMérieux) at the Anaerobe Laboratory, Department of Microbiology, University of São Paulo. All of the organisms were stored in 10% skim milk at −80°C.

Bacterial susceptibility for cefoxitin (Merck, Sharp & Dohme, São Paulo, Brazil) was performed in duplicate by using an agar dilution method in Wilkins-Chalgren agar [12]. Reference strain *B. fragilis* ATCC 25285 was used as control, and the breakpoint used for cefoxitin was 16 μg/ml [13]. Media with antibiotic concentrations ranging from 0.25 to 512 μg/ml and without drug were used. Bacterial inocula were standardized to approximately 1.5×10^8 CFU/ml. Media were inoculated by using a Steer's replicator delivering final inocula of *c.* 1.5×10^5 colony-forming units/spot. Plates were incubated under anaerobic conditions at 37°C for 48 hours. The minimal inhibitory concentration (MIC) value was defined as the lowest concentration of cefoxitin able to inhibit the macroscopic bacterial growth.

Plasmid DNA from all tested strains was obtained by using a Perfectprep Plasmid Mini Kit (Eppendorf AG, Hamburg, Germany). DNA was analyzed by electrophoresis on 0.8% agarose gel. A plasmid-positive *E. coli* J53 pACYC 184 strain was used as control. Plasmid-positive *B. fragilis* species were cured by exposition to different ethidium bromide (EtBr) concentrations (from 0.25 to 64 μg/ml) in brain heart infusion (BHI) broth. Plasmid DNA from 10 colonies randomly selected was extracted and analyzed by agarose gel electrophoresis to verify plasmid loss.

Cefoxitin-resistant bacteria were evaluated to verify the β-lactamase production by a nitrocefin method (Oxoid Ltd, São Paulo, Brazil) using a bacterial colony dissolved in a drop of nitrocefin. After incubation at room temperature for 30 minutes, β-lactamase activity produced a red color. In addition, a biologic method was used to verify cefoxitin hydrolysis according to Nakano et al. [8] as follows: 20 μl cefoxitin-resistant *B. fragilis* were inoculated, in duplicate, onto BHI agar containing 32 μl/ml cefoxitin. After incubation (anaerobiosis, 37°C at 48 hours), the growth was exposed to chloroform vapors for 20 minutes. Then, 3 ml BHI (0.7% agar) inoculated with *Actinomyces viscosus* ATCC 910144, susceptible to cefoxitin (MIC $\leq$ 0.25 μg/ml,) was pour plated. Plates were incubated under anaerobic conditions at 37°C for 72 hours, and the presence of a growth halo over the killed bacteria was considered as producing β-lactamase. β-lactamase-positive *B. fragilis* ATCC 43858 was used as control.

Moreover, the presence of the *cepA* gene, related to β-lactamase production, was evaluated by polymerase chain reaction (PCR) from all purified total DNA and plasmids. Total DNA was obtained as follows: Bacteria were centrifuged (10,000 × *g* for 10 minutes), and the pellet was resuspended in 300 μl lysis buffer (40 mM Tris-acetate at pH 7.8; 20 mM sodium acetate; 1 mM ethylenediaminetetraacetic acid [EDTA] pH 8.0; 1% w/v sodium dodecyl sulfate) and 50 μl of 10 mg ml^{-1} proteinase K, and the tubes were incubated at 37°C for 30 minutes. The extracts were treated with 300 μl chloroform/isoamyl alcohol (24:1), and centrifuged (12,000 × *g* at 4°C). The aqueous phase was saved into a new tube, and 200 μl Tris-EDTA buffer (pH 8.0) was added. The extraction was repeated, and the DNA was precipitated with 500 μl isopropyl alcohol. DNA was resuspended in 50 μl Tris-EDTA buffer (pH 8.0). Aliquots of 20 μl DNA were analyzed by electrophoresis in 2% w/v agarose gel at 90 V for 90 minutes.

Primers for *cepA*, 5′- TTT CTG CTA TGT CCT GCC C -3′ and 5′- ATC TTT CAC GAA GAC GGC -3′, to provide a specific 780-bp band were used [14]. The reaction mixtures were prepared in total volumes of 25 μl containing 15 ng DNA, 0.5 U *Taq* Platinum polymerase (Invitrogen, São Paulo, Brazil), 200 μM deoxynucleoside triphosphate, 1 × PCR buffer, 1.5 mM $MgCl_2$, and 0.4 μM each primer. The PCR conditions were as follows: denaturing at 94°C for 30 seconds, annealing at 55°C for 1 minute, and elongation at 72°C for 5 minutes. The cycles were repeated 35 times. Water and DNA from isolate susceptible to cefoxitin were used as negative controls. All PCR amplification products were detected on 1.5% agarose gel stained with ethidium bromide (0.5 μg/ml).

Results

From the 161 bacteria of the *B. fragilis* group analyzed, 47 (29.2%) were cefoxitin resistant and showed MIC values ranging from 32 to >512 μg/ml. Among them, 7isolates (15%) harbored plasmids, and 3 different plasmid profiles were observed: 5.0, 6.0, and 5.5 kb (Table 1). The other 40 species that were cefoxitin resistant and without plasmid *B. fragilis* appeared have chromosomally mediated resistance as proposed by Pestana et al. [15]. In contrast, from the 47 tested *B. fragilis* group species, 33 (70.2%) were β-lactamase positive by using nitrocefin method; however, when the biologic method was used, no cefoxitin-resistant strain hydrolyzed the drug.

Moreover, only two isolates *B. vulgatus* Bd26e (diarrhea) and *B. fragilis* Bc5j (nondiarrhea) harbored the 5.5-kb plasmids, which were lost after EtBr treatment, and their resistance to cefoxitin decreased from 256 to 4 μg/ml and from >512 to 2 μg/ml, respectively.

The presence of the *cepA* gene conferring resistance to β-lactam antibiotics was detected by PCR amplifi-

Table 1. MIC values for cefoxitin, β-lactamase production, plasmid profile, and presence of *cepA* gene in seven *B.fragilis–group* species isolated from calves with diarrhea

Isolates	MIC Cefoxtin[a]	β-lactamase production	Plasmid band (kb)	*CepA*	
				Chromosome	Plasmid
B. vulgatus Bc11b	64	+	5.0; 6.0	+	–
B. fragilis Bd6d	>512	–	5.0; 6.0	+	–
B. fragilis Bc5j	>512	+	5.5	–	+
B. vulgatus Bc6a	32	+	5.0; 6.0	+	–
B. vulgatus Bc6b	128	+	5.0; 6.0	–	–
B. vulgatus Bc7f	64	1	5.0; 6.0	–	–
B. vulgatus Bd26e	256	+	5.5	–	+

[a]Breakpoint used for cefoxitin, 16 μg/ml.
+, presence; –absence.

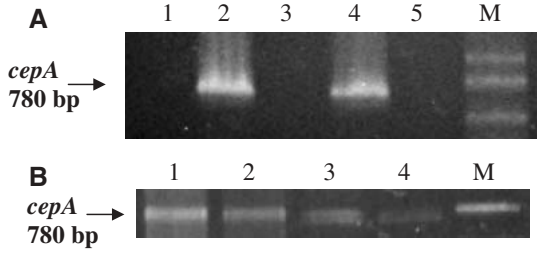

Fig. 1. Amplification of the *cepA* gene from plasmid and total DNA of *B. fragilis*. (A) Lane 1 = *B. fragilis* Bc5j total DNA. Lane 2 = 5.5-kb plasmid from *B. fragilis* Bc5j. Lane 3 = *B. fragilis* Bd26e total DNA. Lane 4 = 5.5-kb plasmid from *B. fragilis* Bd26e. Lane 5 = negative control. Lane M = 1 kb plus DNA ladder. (B) Lane 1 = *B. fragilis* ATCC 43858 total DNA. Lane 2 = *B. fragilis* Bd6d total DNA. Lane 3 = *B. vulgatus* Bc11b total DNA. Lane 4 = *B. vulgatus* Bc6a total DNA. Lane M = 1 kb plus DNA ladder.

cation using specific primers on total DNA from 22 isolates (47%) and two 5.5-kb plasmids, but not on the other 5.0- and 6.0-kb plasmids (Fig. 1). The reference strain *B. fragilis* ATCC 43858 also harbored the *cepA* gene.

Discussion

These results suggest a correlation between resistance to cefoxitin and the presence of the 5.5-kb plasmid. Moreover, it appears to be that a *cepA* homolog may be present in the chromosome and the 5.5-kb plasmids; however, the loss of this plasmid produced a decrease in resistance to cefoxitin. However, residual β-lactamase production was observed by nitrocefin method, but it was not observed by biologic method (data not shown). This result suggests that resistance to cefoxitin in *B. fragilis* could be caused by other mechanisms, such as alterations in both penicillin-binding proteins and outer-membrane proteins [4]. Additionally, *B. fragilis* strains can carry out silent genes such as *cfiA, cepA*, and *cfxA* [6].

The *cfxA* gene harbored in a mobilisable transposon (*Tn*4555) encodes the broad-spectrum CfxA β-lactamase, which can produce high-level resistance to cefoxitin and other β-lactams [5]. In contrast, several classes of β-lactamase enzymes have been described in *B. fragilis* [18]. Most of them are cephalosporinases that are inhibited by clavulanate and are frequently codified by *cepA* or rarely by the *cfxA* gene [16].

Nakano et al. [8] observed the presence of 1.8- and 7.8-kb plasmids in five *B. fragilis* strains, and three of them harbored a 6.4-kb plasmid, which was responsible for the resistance to penicillin and amoxicillin/clavulanic acid by carrying the *cfiA* gene.

The presence of low molecular-weight plasmids is frequently observed in *Bacteroides* spp. Surveys have reported that 20% to 50% of clinical and fecal isolates possess plasmids ranging in size from 2.7 to > 80 kb [17].

In this study, plasmids with low molecular weights were observed, and their presence was correlated to cefoxitin resistance. However, the occurrence of small cryptic plasmids in *Bacteroides* strains in different countries may be due to their horizontal mobilization by different conjugative elements, which may promote their spread through the indigenous microbiota [18]. Data observed in this study are important because *B. fragilis* isolated from cattle carrying resistant determinants to an antimicrobial agent can produce the intestinal microbiota that transfer these genetic elements either to cattle farmers or owners. Because those organisms can acquire antibiotic resistance or pathogenicity determinants, it may render treatment of bacteroides infections more difficult.

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