Occurrence of enterotoxigenic and nonenterotoxigenic *Bacteroides fragilis* in calves and evaluation of their antimicrobial susceptibility

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Abstract

Bacteroides fragilis is considered an important clinical pathogen and the most common anaerobe isolated from human and animal clinical specimens; enterotoxigenic strains produce diarrhea. The presence of enterotoxigenic (ETBF) and nonenterotoxigenic *B. fragilis* in stool samples from calves with or without acute diarrhea and the antimicrobial susceptibility of the strains were evaluated. The stool samples were plated onto a selective *B. fragilis*–bile–esculin agar, and incubated anaerobically (10% CO_2/90% N_2), at 37 °C, for 72 h. Species of the *B. fragilis* group were identified by using the API 32-A kit. Enterotoxigenic strains were detected by PCR and the cytotoxic assay. From 54 diarrhea and 54 nondiarrhea stools, 124 and 92 members of the *B. fragilis* group, respectively, were recovered. Only two ETBF strains were isolated from two different diarrhea samples and the *bft* gene was detected in both. Moreover, the *bft* gene was detected in DNA from four different diarrheal stools samples but no ETBF strain was recovered. All the bacteria were susceptible to chloramphenicol, imipenem, moxifloxacin, piperacillin/tazobactam, metronidazole and tigecycline. Most of the isolates from both calves with and without diarrhea were resistant to all metals. Our results are of concern, and suggest the need to increase the surveillance of antibiotic and metal resistance of this microbial group isolated from animal production such as calves.

Introduction

Species of the genus *Bacteroides* are important constituents of both human and animal intestinal microbiota, comprising approximately 30% of the total of cultured microorganisms from feces (Myers *et al.*, 1987; Moraes *et al.*, 2000). *Bacteroides fragilis* is considered an important clinical pathogen and it is the most common anaerobe isolated from human clinical specimens producing endogenous infections (Duerden, 1994). Moreover, *B. fragilis* is also considered to be the most virulent species in the genus *Bacteroides* due to its virulence factors and its predominance in monomicrobial and mixed infections such as brain, lung, intra-abdominal and intrapelvic abscess, peritonitis and sepsis (Duerden, 1980).

Some strains of *B. fragilis* (ETBF) produce an enterotoxin that causes diarrhea in animals and it has also been associated with diarrhea in humans (Border *et al.*, 1985; Wu *et al.*, 2002). This extracellular toxin has been characterized as a heat-labile zinc-metalloprotease of approximately 20 kDa and is a member of the matrix metalloprotease subfamily of the metzincin superfamily (Moncrief *et al.*, 1995; Wu *et al.*, 2006).

Acute diarrhea in calves within the first months of life has become a serious economical problem worldwide, and it can cause severe damage to the national livestock, due to the delay in development of growing and the high morbidity and mortality rate of infected animals. Classic agents producing diarrhea in calves include enterotoxigenic *Escherichia coli* (ETEC), rotavirus and *Cryptosporidium* (Al-Majali *et al.*, 2000; Yagita *et al.*, 2001). However, little is known about the role that intestinal anaerobes, such as members of the *B. fragilis* group, play in diarrhea processes (Finegold, 1993).

Animals such as cows, sheep and goats are used in milk production or are consumed as meat. However, the practise of feeding animal meat and bone to cattle has been linked to epidemics of bovine spongiform encephalopathy. Not only are harmful prions found in the consumed animal products,

but so too are heavy metals and persistent organic pollutants. For example, metals such as copper are used extensively as a growth-promoting agent in both industrial and organic animal production (Dórea, 2004; Hasman *et al.*, 2006). Substances that are resistant to degradation and that are released in the environment accumulate in the food web and often end up being consumed by humans. The chemical characteristics of these substances determine the location of accumulation, the metabolism and the half-life within animal tissues (Dórea, 2004).

Members of the *B. fragilis* group have been studied because of their pathogenic potential and because of their unusual multiple resistance to several antibiotics and heavy metals (Salyers, 1984; Avila-Campos *et al.*, 1991; Nakano & Avila-Campos, 2004), which can be transferred within and between species, including gram-negative facultative anaerobic bacteria, such as *E. coli* (Salyers & Amábile-Cuevas, 1997; Gupta *et al.*, 2003).

In this study, the occurrence of enterotoxigenic and nonenterotoxigenic species of the *B. fragilis* group in calves with or without diarrhea was evaluated, as well as the susceptibility of these isolates to antibiotics and metal ions.

Materials and methods

Animals and sample collection

One hundred and eight female calves aged between 7 and 90 days from five farms around the cities of São Paulo and Ribeirão Preto (São Paulo, Brazil) were examined. Calves drank water *ad libitum* and none of them had been treated with antibiotics prior to 1 week before sample collection. Calves with and without acute diarrhea were selected for this study and sex was not used as a selection criterion. Stool samples were collected from 54 calves with diarrhea and from 54 healthy ones, using a sterilized rectal swab, transported in Cary-Blair medium with 0.5% L-cysteine-HCl, and processed within 48 h. One stool sample was taken from each animal. The Ethics Commission for Animal Research of the Institute of Biomedical Sciences of the University of São Paulo approved this study.

Bacterial isolation

Stool samples were plated onto a selective *B. fragilis*–bile–esculin agar (BBE) (Livingston *et al.*, 1978), and incubated in 90% N_2+10% CO_2, at 37 °C, for 72 h. One to three colonies from each stool sampled were subcultured on trypticase soy agar supplemented with 0.5% yeast extract, 5 mg mL^{-1} hemin, 1 mg mL^{-1} menadione and 5% defibrinated sheep blood and then identified by using the API 32-A kit (bioMérieux). All the bacteria belonging to the *B. fragilis* group were stored in 10% skimmed milk at − 80 °C.

Cytotoxicity assays

All the *B. fragilis* group species were assayed for cytotoxic effect on HT-29/C_1 cells, as described by Nakano & Avila-Campos (2004). Briefly, HT-29/C_1 cells were grown in 25-cm^2 flasks with 5 mL of Eagle's medium with glutamine (Difco) supplemented with penicillin (100 UI mL^{-1}), streptomycin (100 μg mL^{-1}) and inactivated fetal bovine serum (15%); they were then incubated in air plus 5% CO_2, at 37 °C. These cells were distributed (200 μL well^{-1}) into a 96-well microtitration plate (Corning, US), and they were allowed to grow for 2–3days. Before the assay, the medium was removed and 180 μL of fresh medium without serum was added, and then 20 μL of the supernatant was added to each well, in duplicate. The plate was incubated at 37 °C, in air plus 5% CO_2, and then examined after 3–4 h, in which the typical toxin-induced cytotoxic changes occur (Pantosti *et al.*, 1997). *Bacteroides fragilis* ATCC 43858, an enterotoxin-positive strain, was used as a control.

bft gene detection

One colony was mixed with 500 μL of Milli-Q ultrapure water and centrifuged twice at 12 000 ***g*** for 10 min. The pellet was resuspended in 300 μL of ultrapure water and boiled for 20 min. After centrifugation (14 000 ***g***, 10 min), the supernatant was used as template in a PCR assay. Moreover, 500 mg of each stool sample was washed twice with ultrapure water and processed as described above.

PCR assays were performed using a specific primer pair according to Pantosti *et al.* (1997): 5′-GACGGTGTATGT GATTTGTCTGAGAGA-3′ and 5′-ATCCCTAAGATTTTAT TATCCCAAGTA-3′ (Invitrogen do Brasil Ltd., São Paulo, SP, Brazil), and amplified a characteristic 294-bp fragment. Amplifications were performed in 25-μL volumes containing 10 × PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP mixture (Invitrogen), 0.5 U *Taq* DNA polymerase (Invitrogen), 0.4 μM each primer and 10 μL of template DNA.

Amplification was performed in a thermal cycler (Perkin Elmer, Amp PCR System 2400), programmed for 94 °C (5 min) followed by 35 cycles of 94 °C (1 min), 52 °C (1 min) and 72 °C (1 min), and then 72 °C (5 min) to allow DNA extension. A negative control without template was included in each PCR run. Amplified products were analyzed by electrophoresis in 1% agarose gel in 1 × TBE buffer (1 M Tris, 0.9 M boric acid, 0.01 M EDTA, pH 8.4) at 80 V for 2 h and then, stained with ethidium bromide (0.5 μg mL^{-1}) and photographed on a UV light transilluminator (Electrophoresis Documentation and Analysis System 120, Kodak Digital Science). A 1-kb DNA ladder molecular marker (Invitrogen) was used. Strains producing cytotoxic changes on HT-29/C_1 cells and harboring the *bft* gene were defined as ETBF.

Antibiotic and metal ion susceptibility testing

The antibiotics used were as follows: amoxicillin, ampicillin, clindamycin and tetracycline (Luper Ind. Farm. Ltd., São Paulo, SP, Brazil); cefoxitin and imipenem (Merck, Sharp & Dohme, São Paulo, SP), penicillin G (Prodoti Lab. Farm. Ltd., São Paulo, SP), tigecycline and piperacillin-tazobactam (Wyeth, Philadelphia, PA), chloramphenicol (Sigma-Aldrich Química, SA, São Paulo, SP), moxifloxacin (Bayer Corporation, West Haven, CT) and metronidazole (Aventis Farm. Ltd., São Paulo, SP). The metals used were as follows: mercuric chloride ($HgCl_2$), silver nitrate ($AgNO_3$), copper sulfate ($CuSO_4$) and nickel sulfate ($NiSO_4.6H_2O$) (Labsynth Prod. Lab. Ltd., São Paulo, SP), cadmium sulfate ($3CdSO_4.8H_2O$) and lead chloride ($PbCl_2$) (Vetec Química Fina Ltd., São Paulo, SP).

Antibiotic susceptibility tests were performed according to CLSI (2007) by using an agar dilution method with Wilkins-Chalgren agar. Plates containing twofold serial dilutions of antimicrobial agents ranging from 0.25 to 512 $\mu g\,mL^{-1}$ were inoculated with a Steers replicator given 1.5×10^5 CFU per spot. Plates without antibiotics or metals were used as controls. All the plates were incubated anaerobically, at 37 °C for 48 h. The minimal inhibitory concentration (MIC) was defined as the lowest concentration of each antimicrobial agent able to inhibit the macroscopic bacterial growth. The antibiotic breakpoints were according to CLSI (2007) and that for metals (2 $\mu g\,mL^{-1}$) according to Riley & Mee (1982) and Avila-Campos *et al.* (1989). Reference strains *B. fragilis* ATCC 43858 and *B. fragilis* GAI 97124 were included in the experiments to assess the reliability of the method, and all tests were performed in duplicate.

β-lactamase production

Penicillin-resistant isolates were tested to verify β-lactamase production by using hydrolysis of the chromogenic cephalosporin (nitrocefin method; Oxoid Ltd., São Paulo, SP). Briefly, a drop of nitrocefin was deposited on a microscope slide and a colony was dissolved on it. The mixture was then incubated at room temperature for 30 min. β-lactamase activity was expressed as negative when no color alterations were observed, and positive when a red color was noted. *Bacteroides fragilis* ATCC 43858 (β-lactamase-positive) was used as control.

Results

Members of the *B. fragilis* group were observed in 37 (68.5%) of the 54 diarrhea and in 30 (55.5%) of the 54 nondiarrhea calves. A total of 216 bacterial isolates were recovered, 124 from calves with diarrhea and 92 isolates from normal stool samples. Table 1 lists the prevalence of species of the *B. fragilis* group in diarrhea and nondiarrhea stools. *Bacteroides vulgatus*, *B. fragilis* and *Bacteroides distasonis* were the most predominant in both calve groups. *Bacteroides eggerthii* was observed in both diarrhea and nondiarrhea samples. Only two ETBF were cytotoxic to HT-29/C_1 cells, both recovered from diarrhea stools. *Bacteroides thetaitaomicron*, *Bacteroides ovatus*, *Bacteroides uniformis* and *Bacteroides caccae* were observed only in calves with diarrhea. They also harbored the *bft* gene (Fig. 1). The *bft* gene was detected in four diarrhea samples, but no alteration on HT-29/C_1 cells was produced (data not shown). From these four stool samples no ETBF strain was isolated.

Table 1. Occurrence of members of the *Bacteroides fragilis* group isolated from calves with diarrhea (54) and without diarrhea (54)

Species	Calves with diarrhea [*n* (%)]	Calves without diarrhea [*n* (%)]
B. vulgatus	63 (50.8)	36 (39.1)
B. fragilis	27 (21.8)	41 (44.6)
B. distasonis	20 (16.2)	8 (8.7)
B. thetaiotaomicron	4 (3.2)	0
B. eggerthii	4 (3.2)	7 (7.6)
B. ovatus	2 (1.6)	0
B. uniformis	1 (0.8)	0
B. caccae	1 (0.8)	0
ETBF	2 (1.6)	0
Total	124	92

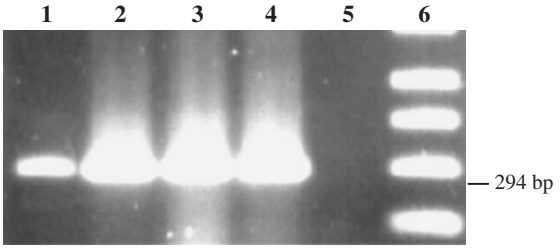

Fig. 1. *bft* gene amplification in bacteria and a stool sample. Lanes 1 and 2, *Bacteroides fragilis* strains B531 and B541, respectively; lane 3, stool sample (B68); lane 4, *B. fragilis* ATCC 43858; lane 5, *Bacteroides uniformis* (negative control); lane 6, 1-kb DNA ladder.

The MIC values and percentage antibiotic and metal resistance are given in Tables 2 and 3, respectively. Of the 216 isolates tested, 161 (74.5%) were resistant at least to one of the β-lactam antibiotics tested (amoxicillin, ampicillin, cefoxitin or penicillin G). In addition, 123 (76.4%) of the 161 β-lactam-resistant isolates produced the enzyme β-lactamase. All 216 isolates were susceptible to chloramphenicol, imipenem, moxifloxacin, piperacillin/tazobactam, metronidazole and tigecycline. Also, 96.7% of the nondiarrhea and 94.3% of the diarrhea isolates were susceptible to clindamycin and tetracycline, respectively.

Most of the isolates tested were resistant to all the metallic ions investigated. Bacteria isolated from calves with

Table 2. Antibiotic susceptibility of 216 members of the *Bacteroides fragilis* group isolated from calves with (124) and without (92) diarrhea

	MIC ($\mu g\,mL^{-1}$)			
Antibiotic	Range	50%	90%	Resistance (%)
Calves with diarrhea				
Amoxicillin	$\leq$ 0.25–$\geq$512	8	512	45.5
Ampicillin	$\leq$ 0.25–$\geq$512	8	128	67.5
Cefoxitin	$\leq$ 0.25–$\geq$512	8	$\geq$512	6.5
Clindamycin	$\leq$ 0.25–64	$\leq$ 0.25	32	25.2
Chloramphenicol	$\leq$ 0.25–4	1	1	0
Moxifloxacxin	$\leq$ 0.25–1	0.5	1	0
Metronidazole	$\leq$ 0.25–16	$\leq$ 0.25	16	0
Imipenem	$\leq$ 0.25–1	$\leq$ 0.25	$\leq$ 0.25	0
Penicillin G	$\leq$ 0.25–$\geq$512	4	16	61.0
Piperacillin/tazobactam	$\leq$ 0.25–1	1	1	0
Tigecycline	$\leq$ 0.25	$\leq$ 0.25	$\leq$ 0.25	0
Tetracycline	$\leq$ 0.25–64	$\leq$ 0.25	8	5.7
Calves without diarrhea				
Amoxicillin	$\leq$ 0.25–$\geq$512	16	512	41.7
Ampicillin	$\leq$ 0.25–$\geq$512	8	64	60.4
Cefoxitin	$\leq$ 0.25–$\geq$512	8	64	25.3
Clindamycin	$\leq$ 0.25–$\geq$512	$\leq$ 0.25	1	3.3
Chloramphenicol	$\leq$ 0.125–4	1	1	0
Moxifloxacxin	$\leq$ 0.125–1	0.5	1	0
Metronidazole	$\leq$ 0.25–16	4	16	0
Imipenem	$\leq$ 0.25	$\leq$ 0.25	$\leq$ 0.25	
Penicillin G	$\leq$ 0.25–$\geq$512	4	32	68.1
Piperacillin/tazobactam	$\leq$ 0.125–1	1	1	0
Tigecycline	$\leq$ 0.125–0.25	$\leq$ 0.125	0.25	0
Tetracycline	$\leq$ 0.25–64	8	32	27.5

Table 3. Metal ion susceptibility of 216 members of the *Bacteroides fragilis* group isolated from calves with (124) and without (92) diarrhea

	MIC ($\mu g\,mL^{-1}$)			Resistance
Heavy Metal	Range	50%	90%	(%)
Calves with diarrhea				
Mercuric chloride	$\leq$ 0.25–32	0.5	4	26.8
Lead chloride	$\leq$ 0.25–$\geq$512	256	$\geq$512	76.4
Silver nitrate	$\leq$ 0.25–32	2	8	47.9
Cadmium sulfate	$\leq$ 0.25–$\geq$512	8	128	62.6
Copper sulfate	$\leq$ 0.25–256	16	128	63.4
Nickel sulfate	$\leq$ 0.25–$\geq$512	32	$\geq$512	67.5
Calves without diarrhea				
Mercuric chloride	$\leq$ 0.25–64	2	4	34.0
Lead chloride	$\leq$ 0.25–$\geq$512	512	$\geq$512	81.3
Silver nitrate	$\leq$ 0.25–64	8	16	83.5
Cadmium sulfate	$\leq$ 0.25–$\geq$512	8	32	61.5
Copper sulfate	$\leq$ 0.25–512	64	256	84.6
Nickel sulfate	$\leq$ 0.25–$\geq$512	128	$\geq$512	93.4

diarrhea showed low levels of resistance to mercuric chloride (26.8%) and but higher resistance (76.4%) to lead chloride. Moreover, bacteria isolated from calves without diarrhea showed low levels of resistance to mercuric chloride (34%) and high levels of resistance (93%) to nickel sulfate (Table 3).

Discussion

No studies on the presence of members of the *B. fragilis* group in farm animals, particularly in calves, have been undertaken in Brazil. Our results show a high prevalence of *B. fragilis* group species in these animals, which could represent an endogenous source in different anaerobic infections (Duerden, 1980).

The presence of ETBF as an agent of acute diarrhea in animals has been observed in several countries. The presence of *B. thetaiotaomicron*, *B. vulgatus*, *B. uniformis*, *B. caccae* and ETBF strains observed only in calves with diarrhea could represent a possible bacterial selection or alteration in the intestinal microbiota in diarrhea processes. Additionally, we observed that DNA obtained from four stool samples from calves with diarrhea harbored the *bft* gene; however, no ETBF strain was isolated from these samples.

The PCR method is an excellent tool for detecting bacterial species from mixed samples. One possibility is that ETBF strains were present at nondetectable levels in those

stool samples, as the supernatant from these four samples did not produce alterations on HT-29/C_1 cells. Our results suggest that other enteropathogens, such as, rotavirus, coronavirus, *Salmonella* spp., enteropathogenic *E. coli*, ETEC or *Vibrio cholerae* could be implicated in the diarrhea processes (Myers *et al.*, 1985). These enteropathogens were not evaluated in this study.

Antimicrobial resistance in anaerobic microorganisms is increasing, particularly in those considered as resident microbiota. The use of antibiotics in bovine cattle is avoided because they are a food source for humans. In this study, all the tested bacteria were susceptible to chloramphenicol, imipenem, moxifloxacin, piperacillin/tazobactam, metronidazole and tigecycline, in accordance with Snydman *et al.* (1996), Aldridge *et al.* (2001), Nakano & Avila-Campos (2004) and Rossi & Andreazzi (2006). However, most of the bacteria showed high levels of resistance to most of the antimicrobials tested, and these results may indicate that animals received drugs with food or from soil contaminated with, for example, metal ions. Brazilian studies have reported the antimicrobial susceptibility of species of the *B. fragilis* group isolated from both human and *Callithrix penicillata* marmoset gastrointestinal tracts showing an increase of resistance to several drugs (Avila-Campos *et al.*, 1991; Carvalho *et al.*, 1997; Pestana *et al.*, 1999). The high frequency of isolation and the predominance of *B. fragilis* in calves could also be attributed to variable breeding conditions, such as possible outside runs, nonauthorized use of antimicrobial growth promoters, limited use of antimicrobials for therapy and age at slaughter.

β-lactamase production in anaerobes is the most important mechanism of resistance against β-lactams (Nord & Hedberg, 1990; Rogers *et al.*, 1993). Most of the isolates tested that produced this enzyme were resistant to ampicillin, amoxicillin, cefoxitin and penicillin G. Moreover, most of the enzymes are chromosomally mediated cephalosporinases with activities against many narrow- and broad-spectrum penicillins and cephalosporins (Rasmussen *et al.*, 1997).

The susceptibility of *B. fragilis* isolated from calves may not exactly reflect the susceptibility of human pathological strains. Clindamycin is the primary choice for treatment of infections produced by anaerobic bacteria. The strains tested in this study showed resistance to this antibiotic, particularly those isolated from diarrhea. *Bacteroides fragilis* isolated from humans have been found to be resistant to clindamycin (Nakano & Avila-Campos, 2004; Betriu *et al.*, 2005; Snydman *et al.*, 2005). On the other hand, tetracycline showed more activity against the tested isolates than cefoxitin, although in human isolates cefoxitin appears to be more effective than tetracycline (Paula *et al.*, 2004). Variations in the resistance rates found in this study may be due to the source of the animals and use of antimicrobial agents in different geographic regions (Souza *et al.*, 2000), and these variations may also produce a bacterial selection in animal microbiota.

Clones of mild pathogenicity may contain resistance genes (i.e. tetracycline, β-lactams and clindamycin) transferable to other more virulent *B. fragilis* strains or to other pathogenic bacterial species (Salyers & Amábile-Cuevas, 1997; Shoemarker *et al.*, 2000). Nakano & Avila-Campos (2004) showed that resistance to β-lactams and other antibiotics and metal ions were higher in children with diarrhea isolates in comparison with nondiarrhea isolates. Recently, Almeida & Avila-Campos (2006) reported the presence of the *cepA* gene in a 5.5-kb plasmid responsible for resistance to cefoxitin from *B. fragilis* isolated from calves with diarrhea, and it is suggested that these genetic elements may be transferable to other *B. fragilis* or other bacterial species sensitive to several drugs.

Chloramphenicol was active against all the isolates tested and preserves an excellent activity against anaerobic bacteria. This antibiotic, which has good tissue penetration, can be used for treatment in cases where its benefit exceeds the risks of toxicity (Wyho *et al.*, 2007).

Moxifloxacin has been reported to have activity against a broad spectrum of both aerobic and anaerobic bacteria (Goldstein *et al.*, 1997) and has also been reported to penetrate and accumulate in the human gastrointestinal mucosa (Wirtz *et al.*, 2004). The CLSI breakpoint has yet to be established for moxifloxacin against anaerobic bacteria and in this study we chose a value of $2\,\mu g\,mL^{-1}$ in accordance with Goldstein *et al.* (2006). However, methodologies and media used to evaluate moxifloxacin activity may also differ due to geographic factors, clonal populations and source of the isolates (Goldstein *et al.*, 2006).

Piperacillin/tazobactam remains the most active β-lactamase inhibitor combination. This class of antibiotics remains very active against the *B. fragilis* group. Our data showed that tigecycline was active against all the *B. fragilis* strains tested. This drug exhibits robust activity against bacterial strains resistant to various antibiotic classes, including β-lactams and fluoroquinolones. However, tigecycline has been used as monotherapy for serious infections in human clinical trials, but in animals it has not been used (Bauer *et al.*, 2004).

Heavy metals are widely dispersed in the environment, and inorganic or aggregated chemical substances, such as metalloids and heavy metals, in food represent a severe risk for a population for their long-term toxicologic effects. The toxicity induced by excessive levels of some of these elements, such as cadmium, lead and mercury, are well known (Llobet *et al.*, 2003).

All bacteria showed high MIC values to most metal ions, but mainly to nickel sulfate and lead chloride. Resistance to metal ions has been discussed in bacteria, and it may be

related to antibiotic resistance according to Riley & Mee (1982). Data regarding metal susceptibility in *B. fragilis* group bacteria are few; however, metal ion resistance in bacteria has gradually increased due to the use of metals in industry, agriculture, hospitals and medicine (Nakano & Avila-Campos, 2004). Our results show variable susceptibility to metals among *B. fragilis* isolated from diarrhea and nondiarrhea calves and it may indicate ecologic and physiologic alterations during the diarrheal process.

On the other hand, the bacterial resistance to metal ions may represent possible environmental contamination, such as of soil and pastures, and it must be viewed with caution because cattle serve as food for humans and other animals (Allen, 1992). In addition, food contamination with metals is an ancient concern given that they are transferred quickly through the food chain. Therefore, considering the endogenous origin of these bacteria and the conjugal transfer properties among intestinal bacteria, these results are of concern, and they reaffirm the need to increase our surveillance the antibiotic and metal resistance of this microbial group isolated from animal production.

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