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Original Research

Intestinal *Bacteroides vulgatus* showing resistance to metals

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ABSTRACT

Background: *Bacteroidaceae* species are predominant in human large intestine, particularly in the colon. These microorganisms are able to develop resistance to several antimicrobial drugs. **Aim:** The aim was to determine the resistance profile and presence of genes encoding resistance to metals in intestinal *Bacteroidaceae* from children and calves. **Methods:** A total of 44 strains were tested. Antimicrobial resistance and presence of resistance genes were evaluated. Resistant strains were submitted to plasmid cure, and the susceptibility was tested again. **Results:** Most of the tested strains were resistance to metals and a strain of *Bacteroides vulgatus* (human origin) showed 3.0 kb and 5.0 kb plasmids and a strain of *B. vulgatus* (animal origin) showed a 4.0 kb plasmid in which the presence of genes *czcA, czcB,* and *czcC* was observed. Resistant strains lost the plasmids and resistance to metals was decreased. **Conclusions:** The presence of genes might be any association with the cadmium resistance. Further studies to characterize the resistance mechanisms or CzcCBA efflux system in *Bacteroidaceae* species are necessary.

KEY WORDS: *Bacteroides vulgatus,* gene *czc,* plasmids, resistance to metals

INTRODUCTION

The human gastrointestinal tract contains a numerous and complex microbiota, and *Bacteroidales* species are predominant, particularly in colon [1]. Species of this taxon are often associated to opportunistic mixed infections, such as intra-abdominal, obstetric-gynecologic and diabetic foot infections. These intestinal species carrying plasmids and/or transposons are able to develop resistance to several antimicrobial drugs, including metal ions [2].

The presence of heavy metals in nature is not a recent fact, and their distribution on the terrestrial surface was due to the volcanic activities and others geological events, such as rain, sediments and earthwaques. Nowadays, industries have collaborated for the humans and animals contamination due to the inadequate planning to eliminate the metal residues [3,4]. In addition, household appliances, antiseptics, thermometers can contain mercury, and it could be other human contamination route [3].

Most of the heavy metals are able to form complex compounds with a redox activity and play an important role in bacterial metabolism. In low concentrations, metallic ions such as zinc and cobalt are essential to the microbial growth [3]. On the other hand, other metals such as mercury and cadmium are not beneficial to bacteria because in low concentrations they are able of binding to sulfhydryl and thiol groups producing highly toxic compounds and inhibiting the proteolysis activity [5]. These cations are transported to the cell inside using two uptake systems: (1) A nonspecific system which is constitutively expressed and produces a metal-ion homeostasis; and (2) a highly specific membrane transport system which is an inducible process, and it is used to uptake nutrients of the growth medium. Similar transport mechanisms are produced by bacteria against toxic metals and metalloids [5].

Because highly contaminated environments with metals are often observed, microorganisms displaying resistance to several heavy metals are found [6]. The bacterial resistance to metals is a well-known phenotypic characteristic encoded by genes carried on plasmid or chromosome. The mechanisms of resistance to metallic ions usually involve either an enzymatic detoxification or an efflux pumping system. Efflux pumping has an important role in the bacterial resistance to many drugs, including heavy metals [7,8].

Studies in *Cupriavidus metallidurans* (formerly *Alcaligenes euthrophus*), an aerobic gram-negative bacterium, have shown the presence of two indigenous plasmids pMOL28 (180 kb) encoding the bacterial resistance to nickel and cobalt; and pMOL30 (238 kb) encoding the bacterial resistance to zinc, cadmium, and cobalt [9]. An inducible bacterial resistance produced by Cd^{2+}, Co^{2+} and Zn^{2+} is encoded by the *czc* (*czc*A, *czcB* and *czcC* genes) localized on pMOL30 that form an efflux pump CzcCBA. This efflux pump includes three subunits: (1) CzcA localized at the cytoplasmic membrane as a cation/proton antiporter; (2) CzcB at the periplasm as a membrane fusion protein; and (3) CzcC an outer-membrane protein. These proteins carry cations out of the cell producing a decrease in intracellular cations [10].

The bacterial resistance to metals is also encoded by regulatory genes (*czc*R, *czc*S, and *czcD*) forming the cation diffusion facilitator (CDF) system (CDF family-single polypeptide chemiosmotic efflux system) with unknown mechanism [11]. An active efflux pump the CzcD protein is required for regulation of the operon *czc* expression, but not for the cation transports [4].

On the other hand, in most of the bacteria, the resistance to mercurial compounds is mediated by a mercuric reductase which is an inducible nicotinamide adenine dinucleotide phosphate-dependent enzyme, and the operon *mer* which is comprised by genes *mer*A (encoding the mercuric reductase production), *mer*R and *merD* (regulatory function), may be localized on plasmids, chromosomes or transposons [12].

Because men and animals may be contaminated with metals through food, water, and soil, and because is also possible to observe a crossed bacterial contamination among them, in this study, the resistance profile and the presence of genes encoding the resistance to metal ions in intestinal *Bacteroidaceae* species were determined.

METHODS

Strains

Forty-four strains in the *Bacteroidaceae* stored at the Anaerobe Laboratory from Biomedical Science Institute of the University of Sao Paulo were analyzed. These strains were isolated from children (11 *Bacteroides fragilis* strains, 5 *Bacteroides uniformis*, 3 *Bacteroides vulgatus* and 3 *Parabacteroides distasonis*) and from calves (7 *B. fragilis*, 1 *B. uniformis*, 10 *B. vulgatus*, 1 *Bacteroides eggerthii* and 3 *P. distasonis*) with diarrhea. Bacteria were grown onto *B. fragilis*-bile-esculin agar to check the purity and then, identified again using an established methodology [13]. Reference strains, *B. fragilis* ATCC 25285, *Escherichia coli* pDU1358 (*mer*A- and *merR*-positive), J53 pACYC 184, and *C. metallidurans* CH34 pMOL30 (*czc*A-, *czcB*-, *czc*C- and *czcD*-positive) were used.

Minimal Inhibitory Concentration (MIC) Assays

The antimicrobial susceptibility was performed using an agar dilution method according to Sutter *et al.* [14] with a Wilkins-Chalgren agar. The metals used were: Zinc sulfate ($ZnSO_4$), cadmium sulfate ($CdSO_4$), mercuric chloride ($HgCl_2$) and cobalt sulfate ($CoSO_4$). Plates containing two-fold serial dilutions of metals ranging from 0.25 to 512 μg/mL were used, and final inocula of 1.5×10^5 cfu/spot were delivered by a Steers replicator. Plates without metals were used as controls. All the plates were incubated in anaerobiosis (90% N_2/10% CO_2), at 37°C, for 48 h. The MIC was defined as the lowest concentration of each metal able to inhibit the macroscopic bacterial growth. Reference strain *B. fragilis* ATCC 25285 was included in each experiment to assess the reliability of the method. All the tests were done in duplicate.

Plasmids and Plasmid Curing

Plasmid DNA from each tested strains was obtained using a Miniprep Plasmid kit (Invitrogen do Brasil Ltd., Sao Paulo, SP, Brasil). DNA was analyzed by 0.8% agarose gel electrophoresis. A plasmid-positive *E. coli* J53 pACYC 184 strain was used as control. Plasmid-positive strains were grown in BHI broth with different concentrations (from 1 to 512 μg/mL) of ethidium bromide (EtBr) [15]. Plasmid DNA from five randomly selected colonies were obtained and analyzed on 1% agarose gel in order to verify the plasmid loss and the susceptibility to metals was also evaluated to determine the resistance values.

Detection of Resistance Genes

Bacterial genomic DNA from each strain was obtained using an Easy-DNA kit (Invitrogen) according to the manufacturer's instructions. Specific primer pairs designed here were used in polymerase chain reaction (PCR) assays to detect the presence of genes *mer*A, *mer*R, *czc*A, *czcB*, *czcC*, and *czcD*. All the sequences were retrieved from GenBank and analyzed by multisequence alignment with CLUSTAL-W (http://ebi.ac.uk/tools/msa/clustaw2/). On the basis of the multisequence alignment, six primer pairs were designed [Table 1] and analyzed with a NetPrimer Analysis Software (http://www.premierbiosft.com/netprimer). The specificities of the primers were predicted by comparison to all available sequences using the BLAST database search program (www.ncbi.nlm.nih.gov/BLAST). DNA amplifications were performed in volumes of 25 μL containing 1 × PCR buffer, 50 mM $MgCl_2$, 0.2 mM dNTP mix, 0.4 mM of each primer, 0.5 U *Platinum Taq* DNA polymerase (Invitrogen), and 1 ng of DNA. Amplifications were performed in a thermal cycler (Perkin Elmer Amp PCR System 9700). PCR products were analyzed by 1% agarose gel electrophoresis, stained with EtBr and photographed under UV light. In all PCR assays ultrapure water and DNA from *B. fragilis* ATCC 25285 were used as negative controls.

RESULTS

MIC values of metal ions and the resistance rates of the strains from human and animal origin are shown in Table 2. The resistance values were similar to all the strains from both children and calves. A minimal difference of resistance values to $HgCl_2$ among human (91%) and animal (77%) strains was observed.

Table 1: Oligonucleotide sequences used to detect the presence of genes in *Bacteroidaceae* species

Genes	Oligonucleotides* 5′→3′	Amplification cycles	Amplicon (bp)
merA	GTT TCA AGG ACG ACC GAG AC CCT CGG TCG AAG TCC AGT AG	35 cycles 94°C×60 s 55°C×60 s 72°C×60 s	151
merR	GAT CTC GTC GAG GCT GAA TC TCT GAC TAT TGG CGT TTT CG	35 cycles 94°C×30 s 52°C×30 s 72°C×60 s	190
czcA	TCA TTA GTT TCG CCA TCC A TGA TGC GTC TGA GTG ACT GG	35 cycles 94°C×30 s 53°C×30 s 72°C×4 min	3151
czcB	GAT GGT GGC GTT CTG CT TTC GGC CTT TCA GAA CAA AAC	35 cycles 94°C×30 s 53°C×30 s 72°C×2 min	1468
czcC	GAA ATC CAC CCG TAC ATC GCC GCC TGA TAG GTT TGT C	35 cycles 94°C×30 s 53°C×30 s 72°C×2 min	951
czcD	GAA GTG GTC GGT GGT GTC AT GAC TTC GAT CCG ACC AGT G	35 cycles 94°C×30 s 52°C×30 s 72°C×2 min	840

*All primer sequences were designed in this study

Table 2: Susceptibility to four metal ions of *Bacteroidales* species

Metals	MIC (μg/mL)			Resistance
	Range	50%	90%	Percentage
Human strains (22)				
$HgCl_2$	1-8	2	4	91
$ZnSO_4$	2-≥512	64	≥512	93
$CoSO_4$	1-≥512	256	≥512	95.5
$3CdSO_4.8H_2O$	1-32	8	16	95.5
Animal strains (22)				
$HgCl_2$	1-8	4	8	77
$ZnSO_4$	1-≥ 512	128	256	95.5
$CoSO_4$	2-≥ 512	64	≥512	100
$3CdSO_4.8H_2O$	0.5-64	32	64	91

Breakpoint for all metals was 2 μg/mL. $HgCl_2$: Mercuric chloride, $ZnSO_4$: Zinc sulfate, $CoSO_4$: Cobalt sulfate, $3CdSO_4.8H_2O$: Cadmium sulfate

Totally, 2 out of 44 strains (one from human and one from the animal) harbored plasmids. *Bacteroides vulgatus* from human harbored 3.0 kb and 5.0 kb plasmids and *B. vulgatus* from animal harbored a 4.0 kb plasmid. In Table 2 is observed the MIC values to the wild strains and their respective clones, as well as, the number of plasmids and their respective molecular weight.

Interestingly, plasmid-positive *B. vulgatus* were isolated from a child and a calf, and plasmids were lost after treatment with EtBr. The bacterial susceptibility to metals after plasmid cure was again determined. MIC values decreased to $HgCl_2$ and $CdSO_4$, respectively, *B. vulgatus* strain H40c (4-2 mg/mL) from human strain and *B. vulgatus* strains B2-3i (32-8 mg/mL) from animal [Table 3] were observed.

Gene *merA*, *merR*, *czcA*, *czcB*, *czcC* or *czcD* were not detected on chromosome in any *Bacteroidales* species from human or animal origin. Furthermore, genes *merA* and *merR* were not detected on plasmid from *B. vulgatus* isolated from both origins. Although, genes *czcA*, *czcB*, and *czcC* were observed on plasmid from *B. vulgatus* B2-3i (animal origin) that showed a decreased MIC value to cadmium.

DISCUSSION

Microorganisms belonging to the human and animal intestinal microbiota are constantly exposed to several antimicrobial agents, including heavy metals. It is suggested that resistance to metals is encoded either by chromosomal or plasmid genes. Although, others specific or unspecific mechanisms, such as exclusion by active transport, enzymatic detoxification, intracellular or extracellular sequestration by protein binding, and metal exclusion by permeability can also codify that resistance [16].

In this study, all the *Bacteroidales* species showed a multiple resistance to the tested metals. The resistance values for all the metals varied from 77% to 100% in both bacterial strains (human and animal origin) [Table 2]. The high resistance level of the tested strains to $ZnSO_4$ and $CoSO_4$ was expected, but the resistance to $HgCl_2$ and $CdSO_4$ suggests a constant contamination of soil, water, and food, reaching humans and animals, particularly, in huge cities, as Sao Paulo, SP, Brazil, and it can become a health public problem.

Resistance genes to antimicrobials have often been found on plasmids; however, closed systems are also subsequently observed on chromosome, e.g. in *Bacillus*, the mercury resistance is encoded by chromosomal genes, and to cadmium by efflux pump [8]; and gram-negative bacteria such as *E. coli*, *Salmonella* spp. and *Pseudomonas* spp., harbor plasmids associated with the resistance to mercurial ions [17].

Intestinal *Bacteroidales* species often display genetic elements, such as plasmids and/or transposons, due to their predominant number and because of closed contact with other intestinal bacteria [18]. Clinical isolates of *Bacteroidales* often harbor cryptic plasmids (from 3 to 7 kb of size) with no defined phenotypic or genotypic association [19,20].

In this study, the multiple resistances to metals and the absence of resistance genes (*merA*, *merR*, *czcA*, *czcB*, *czcC* and *czcD*) on chromosome from the evaluated strains, suggest that another mechanism like efflux pumps or plasmid genes may collaborate with this resistance. Bacteria can use efflux pumps in order to regulate the intracellular metal concentration even if they are not able to growth in the environment with high metal concentrations [21]. Ueda *et al*. [22] reported the presence of 16 efflux pumps belonged to the Resistance Nodulation Division Family in *B. fragilis*, called BmeABC (*B. fragilis* multidrug efflux) conferring an intrinsic resistance against antibiotics, detergents, dyes, and biocides. It is known that in metal-resistant bacteria, multiple pumps will be recruited, and the presence

Table 3: Antimicrobial susceptibility and plasmid profile in *B. vulgatus* (wild strains and respective clones) from human and animal origin

Strain/clones	$HgCl_2$ (μg/mL)	$ZnSO_4$ (μg/mL)	$CoSO_4$ (μg/mL)	$3CdSO_4.8H_2O$ (μg/mL)	Plasmid No. (kb)
Human					
B. vulgatus H40c[a]	4	256	≥512	8	2 (3.0; 5.0)
B. vulgatus H40c1[b]	2	256	≥512	8	-
B. vulgatus H40c2[b]	2	128	≥512	8	-
Animal					
B. vulgatus B2-3i[a]	2	64	64	32	1 (4.0)
B. vulgatus B2-3i1[b]	0,5	64	32	8	-
B. vulgatus B2-3i2[b]	1	64	16	8	-
B. vulgatus B2-3i3[b]	1	64	32	4	-
B. vulgatus B2-3i4[b]	2	32	32	8	-
B. vulgatus B2-3i5[b]	1	64	64	8	-

Breakpoint for all metals was 2 μg/mL. [a]Wild strain, [b]Clones from wild strain. *B. vulgatus: Bacteroides vulgatus*

of divalent cations such as cadmium, zinc or cobalt may active the bacterial efflux system; however, the resistance mechanisms against metals remain unclear [2,23].

The 4.0 kb plasmid found in *B. vulgatus* B2-3i (animal strain) was lost after EtBr treatment, and a decreased resistance value was observed. In addition, the *czcA*, *czcB*, *czcC*, and *czcD* genes were detected on this plasmid, and this suggests a relationship with the cadmium resistance [24]. The 4.0 kb plasmid was easily lost, suggesting its great instability; however, sequencing and mutation analysis of this plasmid might be important to better know the cadmium resistance mechanism in this organism.

The presence of resistance genes to different metals in *B. fragilis* has been shown [6]; and here we are reporting the presence of *czc* genes in intestinal *B. vulgatus*. Further studies to characterize the CzcCBA efflux system in *Bacteroidales* species will be necessary.

Moreover, the broad distribution of extra-chromosomal elements may indicate a potential problem, mainly when bacteria harboring resistance genes to antibiotic and/or metals, as well as pathogenicity determinants, may become the anaerobic infections treatment, particularly those produced by *Bacteroidales* species, more difficult in humans and animals. On the other hand, farm animals, such as cattle may be contaminated with metal ions by contact with grasses, water and soils [13].

The presence of intestinal bacterial strains displaying resistance to metals suggests an evident human and animal contamination, and other microorganisms from the intestinal resident microbiota might be evaluated, as well as our results could be used to investigate the presence of toxic metal in the environment.

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