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 eutrophus*), 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 Cd²⁺, Co²⁺ and Zn²⁺ is encoded by the *czc* (*czcA, 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 (*czcR, czcS*, 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 nicotineamide adenine dinucleotide phosphate-dependent enzyme, and the operon *mer* which is comprised by genes *merA* (encoding the mercuric reductase production), *merR* 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 *Bacteroides* species were determined.

**METHODS**

**Strains**

Forty-four strains in the *Bacteroides* species 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 Chalgren agar. The metals used were: Zinc sulfate (ZnSO₄), cadmium sulfate (CdSO₄), mercuric chloride (HgCl₂) and cobalt sulfate (CoSO₄). Plates containing two-fold serial dilutions of metals ranging from 0.25 to 512 μg/mL were used, and final inocula of 1.5 × 10⁶ cfu/spot were delivered by a Steers replicator. Plates without metals were used as controls. All the plates were incubated in anaerobiosis (90% N₂/10% CO₂), 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 *merA, merR, czcA, czcB, czcC*, and *czcD*. All the sequences were retrieved from GenBank and analyzed by multiple sequence alignment with CLUSTAL-W (http://ebi.ac.uk/tools/msa/clustaw2/). On the basis of the multiple sequence alignment, six primer pairs were designed [Table 1] and analyzed with a NetPrimer Analysis Software (http://www.premierbiosoft.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 MgCl₂, 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 HgCl₂ among human (91%) and animal (77%) strains was observed.
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 ZnSO4 and CoSO4 was expected, but the resistance to HgCl2 and CdSO4 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 or 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 of resistance genes (merA, merR, czcA, czcB, czcC or czcD) 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.
of divalent cations such as cadmium, zinc or cobalt may activate 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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REFERENCES


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