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In vitro effect of antibiotics on biofilm formation by *Bacteroides fragilis* group strains isolated from intestinal microbiota of dogs and their antimicrobial susceptibility

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ABSTRACT

The *Bacteroides fragilis* group strains colonize the intestinal tract of dogs as commensal bacteria. Nevertheless, they can be opportunistic pathogens responsible for significant morbidity and mortality rates in dogs, like in oral infections, abscesses and wound infections. The purpose of this study was to evaluate antimicrobial susceptibility in *B. fragilis* strains isolated from dogs intestinal microbiota and to evaluate the effect of subinhibitory concentrations of some antimicrobials on biofilm formation. A total of 30 *B. fragilis* group strains were tested for susceptibility to ten antimicrobial agents by broth microdilution method. Thirteen *B. fragilis* strains were tested for biofilm formation and the biofilm producer strains were chosen to evaluate the effect of subinhibitory concentrations of six antimicrobials on biofilm formation. The isolates were susceptible to amoxicillin-clavulanic acid, metronidazole, imipenem and chloramphenicol. Tetracycline and clindamycin were active against 50% and 33% of the strains, respectively. When biofilm-forming strains were grown in the presence of sub-MICs of imipenem and metronidazole, the inhibition of biofilm formation was observed. In contrast, enrofloxacin at ½ MIC caused a significant increase in biofilm formation in two of four strains examined. In conclusion, the *B. fragilis* group strains isolated were susceptible to most of the antimicrobials tested and the sub-MIC concentrations of imipenem, metronidazole and clindamycin were able to inhibit the biofilm formation.

1. Introduction

Bacteroides fragilis group and *Parabacteroides* microorganisms are anaerobic, bile-resistant, non-spore-forming, gram-negative rods [1]. They are normally commensal bacteria of the gut flora of dogs. Nevertheless, these microorganisms can also be responsible for infections with significant morbidity and mortality rates in dogs, like oral infections, abscesses and wound infections [2–4].

Numerous factors contribute to the ability of *B. fragilis* to persist as commensal in the gut, such as the capacity to use a wide range of dietary polysaccharides, high bile tolerance, capsule formation and the presence of variable surface antigens that allow the bacteria to evade the host's immune responses. The capacity for adhesion and biofilm formation is also important factors [5].

The ability to form biofilm is an attribute of a majority of the microorganisms. A biofilm is a structured consortium of bacteria embedded in a self-produced polymer matrix consisting of polysaccharide, protein and DNA. The biofilm enables the bacteria to survive in hostile environments and increase antibiotic resistance due to restricted penetration of antimicrobials, the heterogeneous metabolic activity of microorganisms contained in biofilm and differences in gene expression patterns compared with planktonic cells [6].

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Recent studies have demonstrated that *Bacteroides* strains from human gastrointestinal microbiota can form biofilm *in vitro*. Biofilm is responsible for a wide variety of infections in veterinary medicine such as pneumonia, liver abscesses, bacterial gastroenteritis, wound infections and mastitis [7–10]. The purpose of this study was to evaluate the antimicrobial susceptibility of *B. fragilis* group and *Parabacteroides* isolates and their ability to form biofilm in the presence of sub-MICs of some antimicrobials.

2. Material and methods

2.1. Strains and samples

From January to June 2011, 30 non-duplicated microorganisms of the *Bacteroides* and *Parabacteroides* genera were isolated from the intestinal tract of 50 healthy dogs. The animals used were in medical appointments (mainly vaccine) from the Veterinary Hospital Unit of the State University of Ceará. The hospital's research ethics committee (N° 10610110-2/57) approved the study. Animals that had undergone antimicrobial chemotherapy during the last 30 days were not included.

Rectal swabs with the feces were collected for each animal. The swabs were inoculated in semi-solid pre-reduced Cary & Blair medium (HiMedia®) and sent to the Bacteriology Laboratory of Federal University of Ceará (UFC). The samples were plated on *Bacteroides* Bile Esculin agar (BBE, HiMedia®) supplemented with gentamycin (100 μg/mL), under anaerobic conditions (90% $N_2$and 10% CO_2) in a jar with Anaerobac® system (Probac®).

2.2. Identification of isolates

Bacterial strains isolated on BBE were examined for oxygen tolerance and bacterial morphology by Gram staining. The identification was determined by the fatty acid profile using gas chromatography system (6850CG, Agilent Technologies®) and the MIDI-Sherlock® software, according to manufacturer's instructions (Agilent Technologies®).

2.3. Determination of minimum inhibitory concentration (MIC) to antimicrobials

The MIC was determined by broth micro-dilution method, according to the CLSI guidelines [11]. The antimicrobial drugs evaluated were: penicillin, amoxicillin-clavulanic acid, cefoxitin, imipenem, clindamycin, ciprofloxacin, enrofloxacin, tetracycline, chloramphenicol and metronidazole (Sigma®). To evaluate the susceptibility we used break points according to CLSI, The *B. fragilis* ATCC® 25285 reference strain was included as control. All tests were performed twice.

2.4. Biofilm formation assays

Biofilm formation assays were performed as described previously using 96-well flat-bottom plate [10]. Thirteen *B. fragilis* isolates were evaluated. Briefly, after a 48 h incubation period of bacterial cultures at 37 °C under anaerobic conditions, the content of each well was removed and the wells were carefully washed 3 times with 200 μL of phosphate-buffered saline (PBS). The plates were dried at 60 °C for 60 min and stained with 100 μL of 0.01% w/v crystal violet solution. Crystal violet was removed after 20 min and the dye bound to the adherent cells was solubilized with 95% ethanol. The optical density (OD540 nm) was determined using an automated microtitre plate reader (Multiscan FC, Thermo Scientific®).

The strains were classified according to their adherence ability into the following categories: non-adherent, weakly adherent, moderately adherent, and strongly adherent, using the classification described by Sproule-Willoughby [12]. The experiments were conducted in triplicate and repeated five different times.

2.5. Influence of antimicrobials on biofilm formation

The strongest 4 biofilm-producing strains were chosen to evaluate the effect of sub-MIC concentrations of some antimicrobials on biofilm formation. The antimicrobials tested were cefoxitin, clindamycin, chloramphenicol, enrofloxacin, imipenem and metronidazole (Sigma®). The concentrations tested were ½MIC and ¼MIC of each antibiotic after 48 h of incubation (under anaerobic conditions at 37 °C). The strains were tested using a 96-well flat-bottom plate and Brucella broth (BD® Company) with the same method described for the biofilm formation. The tests, performed in triplicate, were repeated four different times [10]. The four *B. fragilis* strains in Brucella broth without antibiotics were used as control of biofilm formation. For each strain tested we calculated the average of the four values obtained from OD_{540} in the presence and absence of antimicrobials.

The results of biofilm formation were compared individually using the test one-way ANOVA followed by Bonferroni multiple comparison using GraphPad Prism® version 5.00 for Windows. (P value <0.05 was considered significant).

Furthermore, the biofilm formation after 48 h of incubation was evaluated using a confocal laser scanning microscopy (CLSM). The same strongest 4 biofilm-producer *B. fragilis* strains used in the experiment described above were chosen for evaluation of the sub-inhibitory concentrations (½MIC) of two antibiotics (clindamycin and imipenem). These antimicrobials were chosen due the large resistance of the *B. fragilis* strains and to be used a lot in veterinary medicine.

For the CLSM assays, each well of a 12-well plastic tissue culture plate, with a 13-mm diameter glass coverslip placed on the bottom, was filled with 200 μL of a 24 h-culture (0.5 McF) of each strain and 1.8 mL of Brucella broth and incubated under anaerobic conditions for 48 h at 37 °C. The biofilms grown on the coverslips were fixed with 3.7% paraformaldehyde at room temperature for 30 min and were stained with the LIVE/DEAD® BacLight™ Bacterial Viability Kits (Invitrogen®). Fluorescence from biofilms was documented using a CLSM (Olympus®, using program FV10-ASW and version 01.07).

3. Results

The following species were identified of the 30 isolates from the fecal samples: *B. fragilis* (50%), *Parabacteroides distasonis* (14%), *Bacteroides vulgatus*, *Bacteroides thetaiotaomicron*, *Bacteroides ovatus* (10%, each one), *Parabacteroides merdae* and *Bacteroides eggerthii* ($n = 3$%, each one).

The isolates showed lower susceptibility to penicillin, tetracycline and clindamycin (0%, 50% and 33%, respectively). The strains were uniformly susceptible to amoxicillin-clavulanic acid, cefoxitin, chloramphenicol, imipenem and metronidazole (Table 1).

The MIC_{90} values for enrofloxacin and ciprofloxacin were 2 and 32 μg/mL, respectively. Furthermore, the clindamycin showed the highest MIC_{50} and MIC_{90} values from all isolates.

The *B. fragilis* strains were investigated due to their ability to adhere *in vitro* to plastic tissue culture plates to form biofilm. Among *B. fragilis* isolates studied by the first method, 8 (62%) strains were capable of producing biofilm.

The sub-MIC results of six antibiotics on biofilm formation of *B. fragilis* are shown in Table 2. The cefoxitin, imipenem and