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Short Communication

Virulence factors and biofilm production by isolates of *Bacteroides fragilis* recovered from dog intestinal tracts

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Abstract

Bacteroides fragilis colonizes dog guts both as a commensal and as an opportunistic pathogen. This study aims to evaluate virulence factors of 13 *B. fragilis* strains isolated from dog intestinal tracts and their ability for biofilm formation. Capsules were detected in all the evaluated strains. A total of 61.5% of all strains were biofilm producers. These attributes most likely play an important role in *B. fragilis* persistent colonization in the gut.

Key words: *B. fragilis*, biofilm, virulence factors, dogs.

Bacteroides fragilis colonize the dogs gut both as a commensal and as an opportunistic pathogen. In a comparison study between cultured small-intestinal and fecal microbiota in Beagle dogs, Mentula *et al.* (2005) found that *B. fragilis* strains were the most frequently isolated from the jejunum and the second most frequent Gram negative anaerobic strain in feces, followed by Bacteroides vulgatus. Bacterial resistance to antimicrobial agents and virulence are mechanisms that play an important role in determining the outcome of a bacterial infection. The capsular polysaccharide of *B. fragilis*, is one of the most important virulence determinants in this bacterium (Wexler, 2007). The ability to form biofilms is now seen as a universal attribute of microorganisms (Lemon *et al.*, 2008). The organisms within biofilms are notorious for their resistance to the host's immune response and antibacterial agents compared to their free-living planktonic counterparts, thus negatively influencing the outcome of bacterial infections. Although less research exists about biofilms in animals, they are believed to be involved in many diseases such as pneumonia and enteritis, as well as liver abscesses, wound infections and mastitis infections (Clutterbuck *et al.*, 2007; Jacques *et al.*, 2010). These infections can be caused by environmental organisms as well as by species of bacteria that constitute part of the normal microflora of animals. Through a combination of endogenous and exogenous factors, these generally harmless commensals may become pathogenic (Clutterbuck *et al.*, 2007). In the present study, *B. fragilis* strains isolated from dog intestinal tracts were tested *in vitro* to detect virulence factors, such as capsule formation, hemolysis, B-lactamase activity, hemagglutination and hydrophobicity. *In vitro* assays to determine biofilm formation and susceptibility to antimicrobials were also performed.

From January to May 2011, 13 *B. fragilis* strains were isolated from 50 dog intestinal tract. The animals were from the Veterinary Hospital Unit of the State University of Ceará. The study was approved by the Committee of Ethics on Research (number 10610110-2/57). The animal had undergone antimicrobial chemotherapy during the last 30 days was rejected. The feces were collected by a rectal swab and the swabs were inoculated in semi-solid prereduced Cary & Blair medium and sent to the Laboratory of Bacteriology of The Federal University of Ceará. The samples were processed for isolation of *Bacteroides fragilis* group in *Bacteroides* Bile Esculin agar (BBE, HiMedia) supplemented with gentamycin 100 μg/mL, under anaerobiosis (N_2 90% and CO_2 10%). Bacterial strains isolated from BBE were examined for oxygen tolerance and bacterial morphology by Gram stain. The identification was de-

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termined by Gas Chromatography (6850CG System) using the database and Sherlock® software according to manufacturer guidelines (Agilent Technologies®). Capsules were detected by using a Hiss stain with modifications. Capsule was identified under light microscopy (100x) through verification of a clear area around the bacteria (Nakano and Avila-Campos, 2004). For the temperature experiments aliquots of 0.1 mL of a 48 h-culture were inoculated in 5 mL of fresh BHI (Brain Heart Infusion-OXOID) medium and then incubated in water bath at 60 °C for 30 min, and afterwards for 1 h and also at 100 °C for, 1, 5 and 10 min. After the incubation period, the tubes were incubated under anaerobiosis at 37 °C for 48 h. Bacterial growth was interpreted as a heat-resistance. An inoculated BHI broth, not exposed at heat, was used as control. (Nakano and Avila-Campos, 2004). The hemolysis production was performed on Trypticase Soy Agar (OXOID) enriched with horse blood (5%). Plates were inoculated in duplicate by using a Steer's replicator and then incubated in anaerobiosis at 37 °C for 48 h. The hemolytic activity was identified through the verification of a clear zone around the bacterial growth (Nakano and Avila-Campos, 2004). Dog, sheep and human bloods were used to investigate the haemagglutinating activity of the strains. Organisms were grown in BHI broth, in anaerobiosis, for 24 h at 37 °C, then harvested, pellet and washed (10,000*g*, 5 min) 3 times in PBS. The bacterial suspension was adjusted to 1.5 x 10^8 ufc/mL. Dog, human and sheep erythrocytes collected and stored at a concentration of 10% in Alsever's solution. HA was tested qualitatively by mixing 50 μL of erythrocyte suspension with 50 μL of bacterial suspension in a 96-well microtiter plate (V shaped). Hemagglutination activity titers were expressed through the reciprocal of the highest bacterial dilution showing agglutination activity. The hydrophobicity of the bacterial surface was measured through the interaction with hexadecane (Nakano and Avila-Campos, 2004). Strains grown in BHI broth for 24 h, at 37 °C were washed three times in phosphate-urea-magnesium buffer (PUM) and resuspended to *ca*. 1.5 x 10^8 bacteria/mL. Then, 3 mL of bacterial suspension were mixed with 400 μL of n-hexadecane (Sigma Chemical Co., US) and incubated at 30 °C, for 10 min. After the vortex and the separation of two phases, absorbance (A540 nm) was determined. These tests were performed in triplicate. The minimum inhibitory concentration (MIC) was determined by the agar dilution method, according to CLSI (2007). The antimicrobial drugs tested were: penicillin, clindamycin, chloramphenicol and metronidazole (SIGMA). The reference strain *B. fragilis* ATCC 25285 was included for quality control. In order to perform the iodometric strip method, it was utilized the beta-lactamase strips (OXOID). Biofilm formation assays were performed by two methods: the Congo red agar method (CRA- Freeman *et al.*, 1989) and the Microtiter Plate (MTP) Test (Donelli *et al.*, 2012) using a plastic tissue culture plate (Sarstedt). After 48 h incubation of bacterial cultures at 37 °C in anaerobiosis, the plates were fixed in 60 °C for 60 min and were washed with Phosphate-Buffered Saline (PBS). 100 μL of 0.01% w/v crystal violet was then added. The stain was removed after 20 min and bacteria were solubilized with 95% ethanol and the optical density (OD540) was determined. The OD of each well was measured at 540 nm using an automated microtiter plate reader (Multiscan FC, Thermo Scientific).Experiments were done in triplicate .Two *B. fragilis* strains were chosen to study by confocal microscopy: 25C1, a non- producing biofilm strain and 47C4, a stronger-biofilm- producing strain. In order to investigate by CLSM the ability of single bacterial strains to form biofilm, 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 broth culture of each strain and 1,8 mL of pre-reduced BHI broth supplemented with 1% glucose, and incubated for 48 h at 37 °C. After incubation, the content of each well was removed and the wells were washed carefully 3 times with PBS. Biofilms grown on coverslips were fixed with 3.7% paraformaldehyde at room temperature for 30 min and stained with the LIVE/DEAD® BacLight Bacterial Viability Kits (Invitrogen, Molecular Probes®) by adding in each well of a 12-well plate 3 μL of the dye mixture in 1 mL of distilled water for 15 min at room temperature in the dark (Donelli *et al.*, 2012). The stain was aspirate and the coverslips was gently washed twice with distillate water. Fluorescence from stained biofilms was viewed using a CLSM (Olimpus FV 1.000), the mounted specimens were observed using a 60x lens.

All the 13 *Bacteroides fragilis* strains were catalase positive and temperature sensible. The hemolysin test was negative for all the strains. It was found that all strains tested were encapsulated. Hemagglutination activity for dog's blood was detected in 38.4% of the strains, showing HA titers of 1:1. 40% of them also showed hemagglutination activity utilizing human blood (A+). Hemagglutination activity for sheep's blood was negative for all the strains. The *B. fragilis* strains studied showed very low cell surface hydrophobicity values (7.7%) A total of 8 (61.5%) of the strains were beta-lactamase positive. The isolates were uniformly susceptible to metronidazole and chloramphenicol and penicillin resistant. The resistance rate to clindamycin was 69.2%. By the CRA method, 04 (30.76%) isolates showed black colonies with dry crystalline consistency (high biofilm producer), 04 (30.76%) showed black colonies with intermediate consistency (moderate biofilm producer), 04 (30.76%) showed black colonies with mucoid appearance (weak biofilm producer), and 01 (7.7%) showed pink/bordeaux colored colonies (non-biofilm producers). Among 13 isolates, 8 (61.5%) strains were capable of producing biofilm by the standard method. Comparison of the tests (CRA and MPT) showed that in the CRA test, five isolates were found to be false positive (Table 1).Confocal microscopy showed isolated bacteria and small clus-

ters in the 25C2 non-biofilm producer strain image Figure 1(a). The biofilm producer 44C7 strain was observed as cell aggregates Figure 1(b).

Numerous factors contribute for *B. fragilis* ability to persist in the intestinal tract as the ability to use a wide range of dietary polysaccharides, the high bile tolerance, the capsule and the variable surface antigens which allow for host immune response evasion, the ability of adhesion and biofilm formation (Wexler, 2007). Adherence of bacteria to the surface layer cells of the host enables commensal bacteria and potential pathogens to overcome flushing mechanisms which cleanse mucous membranes. Although adhesion is essential for maintaining members of the normal microflora in their host, it is also the crucial first stage in any infectious disease (Wilson, 2002). The capsular polymer can first protect *B. fragilis* cells from phagocytosis and then, probably when produced in larger amounts, stabilize adhesion of cells to epithelia (Nakano *et al.*, 2008). In this study, all the examined *B. fragilis* strains were encapsulated, and these results are according to other studies (Domingues *et al.*, 1995; Nakano and Avila-Campos, 2004). The *B. fragilis* strains studied showed low hydrophobicity values. This may be due to the capsules that are usually hydrophilic and may contribute to the low hydrophobicity of the strains (Oyston and Handley, 1990). Adhesins or outer membrane proteins have been implicated in hemagglutination reactions (Nakano and Avila-Campos, 2004). In this study 38.4% of the strains were HA-positive to dog blood and some strains also agglutinated human blood. The high clindamycin resistance rate observed in this study (69.2%) might be associated to the wide use of macrolides in veterinary clinics. Recent studies have demonstrated that *Bacteroides strains* from human gastrointestinal microbiota can form biofilms *in vitro* (Guaglianone *et al.*, 2010; Sporule-Willoughby *et al.*, 2010). In clinical settings, bacteria in biofilms are less susceptible to antimicrobial agents and host immune responses, thereby becoming persistent colonizers or sources of chronic infections (Donlan and Costerton, 2002). Donelli *et al.* (2012). In this study 61.5% of the *B. fragilis* strains isolated from dogs intestinal microbiota were biofilm producers when evaluated by the standard method. The classic method most often used to phenotypically detect slime production in staphylococcal species is the CRA plate test (Freeman *et al.*, 1989). Some authors have evaluated the effectiveness of this method with Gram negative bacteria (Dadawala *et al.*, 2010; Murugan *et al.*, 2011). On the CRA method the direct analysis of the colonies formed on the solid medium allows the recognition of biofilm-producer strains and of non-biofilm-producer strains by the color changes analysis. Hence, the assay is not quantitative, but based on a chromatic subjective evaluation. In this study, high producing biofilm strains displayed black colonies with a dry crystalline consistency but the other isolates showed colors and consistency difficult to evaluate. In summary, the results of this study showed that eight *B. fragilis* isolates recovered from dog intestinal microbiota were biofilm producers. All of these strains were encapsulated, 50% showed HA activity and all showed high or intermediary clindamycin resistance. Our experience, as well as that of other researchers (Dadawala *et al.*, 2010; Murugan *et al.*, 2011), confirms that the CRA test is easy to perform and little time consuming. But can be very difficult to read and so, to classify strains. In conclusion, some important virulence factors, like the presence of capsules, were observed in all strains tested and the ability to form biofilm was observed in more than half of B. fragilis strains isolated from dog intestinal microbiota. These attributes most likely play an important role in *B. fragilis* persistent colonization in the gut, as in other clinical settings.

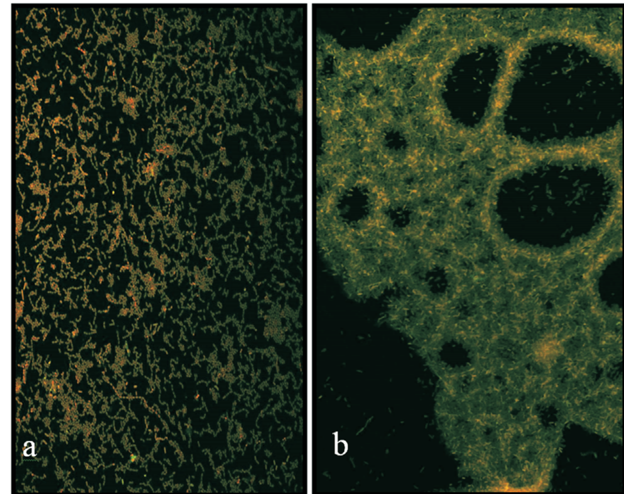

Figure 1 - CLSM micrographs of mono-species biofilm, of non-biofilm producer *Bacteroides fragilis* 25C1 (a) and biofilm of *B. fragilis* 44C7 (b). Biofilms were stained with a live/dead stain.

Table 1 - Screening of biofilm production methods by Congo Red Agar (CRA) and microtiter plate test (MPT).

	Production biofilm	CRA (%)	MPT (%)
Nº strains (13)	Weak	04 (30.7%)	08 (61.5%)
	Moderate	04 (30.7%)	00
	High	04 (30.7%)	00
	Non producers	01 (7.8%)	05 (38.5%)
Total producer strains		12	08

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