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Coincidence of *bft* and *cfiA* genes in a multi-resistant clinical isolate of *Bacteroides fragilis*

Although a common member of the normal human gut flora, *Bacteroides fragilis* possesses various virulence factors, such as capsule, LPS and special surface proteins, and certain types of strains may produce an extracellular enterotoxin, termed fragilysin (BFT). The fragilysin-encoding gene (*bft*) has three different isoforms (termed *bft-1*, *bft-2* and *bft-3*) (Chung *et al.*, 1999; Franco *et al.*, 1997; Kling *et al.*, 1997). Because of the increasing number of resistant *B. fragilis* isolates, the therapeutic possibilities have been restricted to only some antibiotics, such as piperacillin/tazobactam, imipenem, metronidazole, clindamycin and cefoxitin (Rasmussen *et al.*, 1993). Out of these, metronidazole and imipenem are the drugs of choice in the treatment of infections caused by *B. fragilis*; however, the emergence of imipenem or metronidazole resistant isolates has been reported (Rasmussen *et al.*, 1993). Carbapenem and metronidazole resistances are frequently associated with the presence of the *cfiA* gene encoded class B metallo-β-lactamase (Thompson & Malamy, 1990) or various types of 5-nitroimidazole resistance genes (*nimA–G*) (Gal & Brazier, 2004). Strains carrying the *cfiA* gene may show susceptibility to imipenem, because in most isolates this gene is silent, but the presence of insertion (IS) elements in the upstream region of this gene can lead to the development of carbapenem resistance (Podglajen *et al.*, 1992).

By using various molecular typing methods, such as arbitrarily primed-PCR, PFGE and multilocus enzyme electrophoresis, *bft*-positive, *cfiA*-negative strains and *bft*-negative, *cfiA*-positive strains belonged to two different DNA homology groups, and the coexistence of *bft* and *cfiA* genes was not detected (Gutacker *et al.*, 2000; Vallim *et al.*, 2002).

During examination of the prevalence of *bft*- or *cfiA*-positive *B. fragilis* strains isolated from various clinical specimens, we found a multi-resistant *B. fragilis* strain R19811 isolated from a blood culture and identified by rDNA RFLPs in the Anaerobe Reference Laboratory, Cardiff (Wareham *et al.*, 2005). This strain co-harboured the *cfiA* and *bft* genes; therefore, our aim was to characterize its genetic background. *B. fragilis* strain R19811 and control strains were cultured as previously described (Sóki *et al.*, 2000). The list and the description of the control strains have been described before (Sóki *et al.*, 2006).

MICs to a range of antibiotics were determined by using the E-test method (AB Biodisk) according to the manufacturer's instructions. *B. fragilis* strain R19811 was multi-resistant to penicillin G (MIC $\geqslant$256 μg ml^{-1}), amoxicillin/clavulanic acid (MIC $\geqslant$256 μg ml^{-1}), cefoxitin (MIC $\geqslant$256 μg ml^{-1}), imipenem (MIC $\geqslant$256 μg ml^{-1}), clindamycin (MIC $\geqslant$256 μg ml^{-1}) and metronidazole (MIC=64 μg ml^{-1}). Because of the imipenem resistance, the presence of *cfiA* gene encoding metallo-β-lactamase was tested. *cfiA* PCR (Podglajen *et al.*, 1992; Thompson & Malamy, 1990) revealed that the strain carried the *cfiA* gene, at the same time the presence of *bft* gene was also detected by PCR according to Pantosti *et al.* (1997).

We analysed the upstream region of the *cfiA* gene to detect IS elements, hypothesizing that the occurrence of one of these sequences may contribute to the development of carbapenem resistance mechanisms; in *B. fragilis* strain R19811 an insertion was detected in the upstream region of *cfiA* gene by G and Up2 primers, as described by Podglajen *et al.* (1994). To determine the type of this element, various primers, IS*614* (IS614-1i, 5′-GAAATTGTGTATCAATGCCG-3′ and IS614-2i, 5′-CTGATACCATCCTCAGAG-CC-3′), IS*942* (Rasmussen & Kovacs, 1991), IS*1169* (Trinh *et al.*, 1995), IS*1170/1* and IS*1170/2* (Trinh *et al.*, 1995), IS*1186* (Podglajen *et al.*, 1994), and IS*4351* (Podglajen *et al.*, 1994; Rasmussen *et al.*, 1987) were tested for use in PCR. During these amplification reactions, standard PCR reaction mixture was used, as described previously by Sóki *et al.* (2006). Of the examined IS elements, only IS*614* was detected by PCR. Partial nucleotide sequence data of the upstream region of the *cfiA* gene by Up2, G, IS614-1i and IS614-2i primers, as described by Sóki *et al.* (2004), confirmed that it is an IS*614*B element.

B. fragilis strain R19811 contained plasmids with an estimated molecular mass of 5.6 and 9.9 kb using *Hin*dIII restriction enzyme (Pharmacia Biotech) analysis. In spite of the fact that *cfiA* gene could be on a plasmid (Bandoh *et al.*, 1992; Nakano *et al.*, 2004), in our case, Southern blot hybridization, showed it was not.

In spite of the observed metronidazole resistance, *nim* genes commonly responsible for metronidazole resistance in the *B. fragilis* group could not be detected using the nim-3 and nim-5 primer pair (Trinh & Reysset, 1996), which is suitable for the detection for all described *nim* genes. The annealing temperature of *nim* PCR was reduced from 62 to 52 °C to show incidental sequence variation in the *nim* gene, but PCR products could not be detected. Further investigations are necessary to establish the metronidazole resistance mechanism in this organism.

In numerous earlier studies, the presence of the *cfiA* gene was never observed together with the *cepA* gene encoding endogenous class A β-lactamase, because *cfiA*-positive and *cepA*-positive strains belong to two genetically distinct groups. The first group was characterized by the presence of *cfiA* gene, while in the second group, the presence of the *cepA* gene is typical with or without the presence of the *bft* gene. In spite of this observation, in 2005, Ayala *et al.* (2005) described two *B. fragilis* strains (7160 and 213E), which

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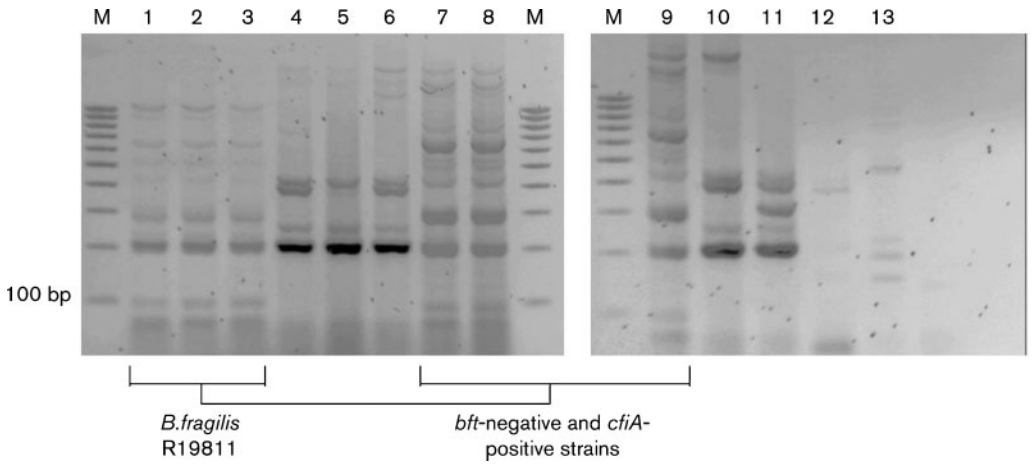

Fig. 1. Comparative ERIC1-2 PCR pattern of *B. fragilis* strain R19811. Two groups of *B. fragilis* strains with similar patterns are indicated: the *cfiA*- and *bft*-positive strain R19811 in triplicate (first group) and *bft*-negative, *cfiA*-positive strains (second group). Lanes 1–3, *cfiA*- and *bft*-positive *B. fragilis* strain R19811; lanes 4–6, *bft*-positive and *cfiA*-negative isolates; lanes 7–9, *bft*-negative strains and *cfiA*-positive strains; lanes 10–11, *bft*-negative and *cfiA*-negative *B. fragilis* strains; lane 12, *Bacteroides thetaiotaomicron*; lane 13, '*Bacteroides variabilis'*. M, 100 bp ladder (Sigma; 100–1000 bp).

possess both the *cepA* and the *cfiA* gene. In strain R19811, no amplification product was detected with *cepA* PCR as described by Gutacker *et al.* (2000). This suggests that the strain originally belonged to the *cfiA*-positive group.

In the cytotoxicity assay, as described by Pantosti *et al.* (1994), *B. fragilis* strain R19811 caused a reversible cytopathic effect on the HT-29 cell line, this result indicated that in the cell culture supernatant, functional toxin, namely fragilysin was present. PCR-RFLP analysis of the *bft* gene by BTT1 (5′-CATGTTCTAATGAAGCTGATTC-3′) and BTT2 (5′-ATCGCCATCTGCTGTTT-CCC-3′) primers, with minor modification according to Chung *et al.* (1999), revealed a *bft*-1 allele.

To clarify the epidemiological background of this strain, enterobacterial repetitive intergenic consensus (ERIC) PCR typing (Versalovic *et al.*, 1991) was applied in the case of *bft*-positive and *cfiA*-negative, *bft*-negative and *cfiA*-positive, *bft*- and *cfiA*-negative strains, and the PCR patterns were compared with the result of ERIC PCR in *B. fragilis* strain R19811. Two homology groups of *B. fragilis* can be distinguished by using molecular typing methods as described previously by Gutacker *et al.* (2000), Moraes *et al.* (1999) and Ruimy *et al.* (1996): the first group is characterized by the presence of the *cfiA* gene, these strains were *bft*-negative, while the strains in the second group carried only the *bft* gene (Fig. 1). ERIC PCR typing suggested that strain R19811, which harboured the *bft* and *cfiA* gene simultaneously, may be related to the first group, which contained only *cfiA*-positive strains (Fig. 1).

In summary, analysis of the results of *cfiA* and *cepA* PCR, and ERIC typing pattern, indicated that *B. fragilis* strain 19811 originally carried the *cfiA* gene and acquired the *bft* gene only later. Franco (2004) described two new related conjugative transposons (CTn*86* and CTn*9343*) in connection with the *bft* gene. Recently, a more divergent picture has arisen. The two conjugative transposons differ in two regions: one, BfPAI, found exclusively on CTn*86* and harbouring the *bft* genes, and the other exclusively harbouring an additional 7 kb region and found on CTn*9343*. However, these two additional regions with the left-end of the transposons may result in hybrid elements. Still, the presence of CTn*86* is mainly characteristic for *bft* genes and the pattern I of *B. fragilis* strains, while CTn*9343* and the CTn*9343*-like elements mainly result in pattern III. The *cfiA* gene, however, was found only in pattern II and some pattern III isolates, but still in division II (Buckwold *et al.*, 2007). If genes responsible for the transfer of these CTns are functional, it is possible that the *bft* gene can be transferred from an enterotoxigenic strain to a nontoxigenic strain. The presence of new transmissible CTns, and virulence and resistance genes, may contribute to the enhancement of the pathogenicity potential and the fitness of this strain; therefore, the aforementioned factors may promote the development of more severe infection, which may be unresponsive to the majority of the therapeutic possibilities.

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Gabriella Terhes,[1] Jon S. Brazier,[2] József Sóki,[1] Edit Urbán[1] and Elisabeth Nagy[1]

[1]Department of Clinical Microbiology, Faculty of Medicine, University of Szeged, H-6725 Szeged, Hungary

[2]Anaerobe Reference Laboratory, NPHS Microbiology Cardiff, University Hospital of Wales, Cardiff, UK

Correspondence: Elisabeth Nagy (nagye@mlab.szote.u-szeged.hu)

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