ANTIMICROBIAL RESISTANCE

Prevalence of the carbapenemase gene (*cfiA*) among clinical and normal flora isolates of *Bacteroides* species in Hungary

J. SÓKI, E. URBAN, I. SZÖKE, E. FODOR and E. NAGY

Department of Clinical Microbiology, Albert Szent-Györgyi Medical University, Szeged, Hungary

The carbapenemase gene (*cfiA*) was detected in 4 (5.7%) of 70 clinical isolates of *Bacteroides fragilis* from different parts of Hungary. Among 24 other *Bacteroides* species isolated from infectious processes or from normal faecal flora, none was *cfiA*-positive. The MIC of imipenem and meropenem for all *cfiA*-positive *B. fragilis* isolates was ≤ 0.25 mg/L, but 17% of the *B. fragilis* and 46% of the non-*fragilis* *Bacteroides* isolates exhibited reduced susceptibility to imipenem (MICs 0.5–2 mg/L). Only one of these isolates produced increased levels of β -lactamase. No difference was observed in the outer-membrane proteins of *B. fragilis* isolates that harboured the *cfiA* gene and those with reduced susceptibility to imipenem.

Introduction

Members of the genus *Bacteroides* (*B. fragilis* group) are frequently isolated from infections after abdominal or gynaecological surgery [1]. These infections are usually polymicrobial and demand the use of potent antibiotics, covering both aerobic and anaerobic pathogens. Increasing resistance of anaerobic bacteria to the antimicrobial agents used for the treatment of anaerobic infections, and differences in the rates of resistance between various hospitals and countries have been reported [2, 3]. Resistance to β -lactam antibiotics in anaerobic bacteria involves the production of β -lactamases, alterations in the penicillin-binding proteins, or the reduced penetration of the antibiotics through the bacterial outer membrane [4]. In *Bacteroides* spp., resistance to β -lactam agents is most often mediated by chromosomal class A β -lactamases with predominant cephalosporinase activity, which are virtually ubiquitous in *B. fragilis* group isolates [5], although the amount of the enzyme varies greatly from isolate to isolate. These enzymes are inhibited by clavulanate, tazobactam and sulbactam and do not hydrolyse cephamycins or carbapenems. Several studies [6–9] have suggested that 0.7–3% of *B. fragilis* strains produce class B carbapenem-hydrolysing metallo- β -lactamases, which are inhibited by ethylenediaminetetraacetic acid (EDTA), but not by the clinically used β -

lactamase inhibitors. The *cfiA* gene coding this enzyme has been cloned and sequenced. It may be ‘silent’ or expressed to various degrees, resulting in a wide range of levels of carbapenem resistance. Some strains harbouring the *cfiA* gene appear sensitive to carbapenems on conventional testing, but can convert to high-level resistance following antibiotic pressure [8]. Clinically relevant *B. fragilis* strains, with or without the presence of the *cfiA* gene, have been reported from different countries to be resistant to imipenem (MIC ≥ 16 mg/L), or to display only a decreased susceptibility (MIC ≥ 1 mg/L) [9, 10]. The present study looked for the presence of the *cfiA* gene by the PCR in clinical isolates of *B. fragilis* collected at three different centres in Hungary. A limited number of strains belonging to other species of *Bacteroides*, obtained from clinical samples or from the normal faecal flora, were also tested.

Materials and methods

Bacterial strains and plasmid

Seventy randomly selected, clinically significant *B. fragilis* isolates and 24 isolates of other *Bacteroides* species (including 10 isolates obtained from faeces of healthy subjects) were collected during 1998 from a large university hospital in south-east Hungary (39 isolates), a large community hospital in the central part of the country (21 isolates) and a small hospital in western Hungary (10 isolates). Isolates were identified by conventional tests or by the Rapid ID 32A system (bioMérieux). *B. fragilis* NCTC 9344 (carbapenem-

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Corresponding author: Dr E. Nagy (e-mail: NAGYE@mlab.szote.U-Szeged.Hu).

sensitive) and *B. fragilis* TAL3636 (a metallo- β -lactamase producer), kindly provided by R. Edwards (Nottingham University), were included as controls. The pJST241 plasmid, carrying the cloned *cfiA* gene [8], was obtained from M. H. Malamy (Boston, MA, USA). The Qiagen Mini Plasmid Purification kit (Qiagen, Hilden, Germany) was used for plasmid isolation.

Antibiotic resistance determination

MICs of carbapenems were determined by the Etest (AB Biodisk, Stockholm, Sweden) on brain heart infusion (BHI) agar supplemented with yeast extract 5 g/L, haemin 5 mg/L and menadione 1 mg/L according to the manufacturer's instructions. MICs were read after incubation for 48 h in an anaerobic environment (Bactron, Shell Lab., Cornelius, USA).

Determination of β -lactamase activity

β -Lactamase activity was determined quantitatively with (0.10 mM) nitrocefin in 50 mM sodium phosphate buffer (pH 7.0, 37°C) by a spectrophotometric method [11]. One unit of β -lactamase was defined as the amount that formed 1.0 μ mole of product/min under the given conditions.

Investigation of outer-membrane proteins

Outer-membrane proteins (OMPs) of selected *B. fragilis* isolates (four strains that were *cfiA*-positive, but imipenem susceptible and four that were *cfiA*-negative, but for which the MIC of imipenem was 1–2 mg/L) were examined by a method based on that described by Carlone *et al.* [12]. The OMPs were solubilised by boiling with 50 μ l of Laemmli sample buffer for 5 min and separated by SDS-PAGE.

*Detection of the *cfiA* gene by PCR*

Bacterial cells from the surface of BHI agar plates were suspended in water and boiled for 10 min. The supernates of the centrifuged suspensions (2 min at 10 000 rpm) were used as template DNA. Reaction mixtures each contained 5 μ l of 10-fold concentrated reaction buffer (Sigma or USB), 1 μ l (2.5 mM) each of dATP, dCTP, dGTP and dTTP (Sigma), 1 μ l (35 pmoles) of each primer, 5 μ l of template DNA, 33 μ l of sterile water and 1 U of Taq polymerase (Sigma). The primers had the nucleotide 557–582 sequence 5'-TCC ATG CTT TTC CCT GTC GCA GTT AT-3' and the complementary 1266–1285 sequence 5'-GGG CTA TGG CTT TGA AGT GC-3' [8]. The reaction mixtures were incubated for 40 cycles in a programmable heating block (GeneAmp, PCR System 9600, Perkin Elmer, Norwalk, CT, USA) for 1 min at 92°C, 2 min at 50°C and 2 min at 72°C with a final extension of 10 min. PCR products were visualised on agarose gels containing ethidium bromide under UV

light and their sizes were compared with those of a mol. wt marker (100-bp DNA ladder, Sigma) and the product generated from *B. fragilis* TAL3636 as a positive control.

Southern blotting with a *cfiA*-specific probe obtained from the PCR product of the pJST241 plasmid was used to confirm the PCR results. PCR products were resolved on agarose 1% gels and transferred to nylon membranes (Amersham, UK) by capillary transfer. Radioactive labelling of the probe was performed with the Megaprime random priming kit (Amersham) under the conditions recommended by the supplier. Pre-hybridisation at 65°C for 1 h was followed by hybridisation at 65°C overnight in 5 $\times$ concentrated saline sodium citrate (SSC) buffer and 5 $\times$ concentrated Denhardt's solution containing sodium dodecyl sulphate (SDS) 0.5% w/v and salmon sperm DNA 100 μ g/ml. Radioactively labelled probe DNA with an activity of *c.* 10⁷ cpm was added to the hybridisation solution after pre-hybridisation. The filters were washed twice in double-strength SSC and SDS 0.1% w/v at 65°C for 15 min, and twice in 0.2 $\times$ SSC and SDS 0.1% w/v at 65°C for 15 min. Damp filters were packed in Saran Wrap and were exposed to X-ray films at –70°C with an intensifying screen.

Nucleotide sequencing

Before nucleotide sequencing, the amplified PCR sample was precipitated with three volumes of 7.5 M ammonium acetate and absolute ethanol (1:5 v:v) at room temperature to remove unincorporated primers. DNA sequencing was performed with an automated sequencer (ABPrism model 373, Applied Biosystems) with an AmpliTaq FS DyeDeoxy terminator cycle sequencing kit (Applied Biosystems). The DNA sequence obtained was compared with those of known carbapenemase genes from *Bacteroides* spp. with the BLAST client programme accessible from the Internet at the National Center for Biotechnology Information.

Results and discussion

The MICs of imipenem for 70 *B. fragilis* and 24 non-*fragilis* isolates ranged from $\leq$ 0.06 to 2 mg/L. No resistant strain was observed according to the NCCLS breakpoint criterion (MIC $\geq$ 16 mg/L) [13]. However, 12 *B. fragilis* isolates (17%) and 11 non-*fragilis* isolates (46%) exhibited reduced susceptibility to imipenem (MICs 0.5–2 mg/L). The *cfiA* gene was detected in four *B. fragilis* isolates and in none of the non-*fragilis* isolates by PCR. Southern blotting of the PCR products revealed homology to the pJST241 probe in *B. fragilis* isolates 20, 72 and 98. The PCR product of *B. fragilis* isolate 22 did not hybridise, but sequence analysis of the product showed that it bore *c.* 96% homology to known *cfiA* genes, indicating that it represents a carbapenemase gene fragment.

Table 1. MICs of carbapenems and the presence of the *cfiA* gene in selected *B. fragilis* isolates obtained from clinical specimens

Isolate	MIC (mg/L) of		<i>cfiA</i>	β -lactamase activity (U/min)
	imipenem	meropenem		
<i>B. fragilis</i> 22	0.06	0.125	+	0.0175
<i>B. fragilis</i> 20	0.06	0.06	+	0.0028
<i>B. fragilis</i> 98	0.125	0.25	+	0.0004
<i>B. fragilis</i> 72	0.25	0.25	+	0.0109
<i>B. fragilis</i> 91	1.0	0.5	–	0.0140
<i>B. fragilis</i> 76	1.0	0.125	–	0.0087
<i>B. fragilis</i> 68	2.0	1.0	–	2.9540
<i>B. fragilis</i> 54	2.0	2.0	–	ND
<i>B. vulgatus</i> 38	1.0	1.0	–	0.0152
<i>B. thetaiotaomicron</i> 55	2.0	2.0	–	0.0185
<i>B. ovatus</i> 42	2.0	2.0	–	ND

ND, not detected.

The MICs of imipenem and meropenem for the four *cfiA*-positive isolates were 0.06–0.25 mg/L (Table 1). Very low β -lactamase activities were measured in these isolates. β -Lactamase activities were also low for three *B. fragilis* and three *Bacteroides* non-fragilis isolates, for which the MIC of imipenem was 1 or 2 mg/L. One strain (*B. fragilis* 68), for which the MICs of imipenem and meropenem were 1 and 2 mg/L, respectively, was found to have high β -lactamase activity. PAGE analysis of the OMPs of the *B. fragilis* isolates with elevated MICs and those that were fully susceptible, but harboured the *cfiA* gene, revealed no difference (results not shown). Similar results have been described by Edwards and Greenwood [14].

No imipenem-resistant isolate has yet been reported in Hungary (the MIC₉₀ was 0.5 mg/L in 1992 and 1 mg/L in 1992–1993), despite the fact that imipenem or meropenem are increasingly used for treatment of severe infections involving aerobic and anaerobic bacteria [10, 15]. Low rates of resistance to imipenem among *B. fragilis* strains have been found in France [2] and in the USA [16], whereas in Japan, where imipenem is widely used, 2.4, 4.5 and 10.5%, respectively, of the *B. fragilis*, *B. thetaiotaomicron* and *B. distasonis* isolates tested were resistant to imipenem [17]. However, the resistance breakpoint used in the Japanese study (6.25 mg/L) was lower than that recommended by NCCLS guidelines (16 mg/L). High-level resistance to imipenem was also found to be rare among *B. fragilis* isolates collected in Nottingham, UK [9], although 12% of 175 *Bacteroides* isolates from clinical specimens exhibited a reduced susceptibility to imipenem (MICs 0.5–2 mg/L, i.e., concentrations up to 50 times higher than those for 'normal' sensitive strains [9]). In the present study, MICs of imipenem for 17% of the *B. fragilis* and 46% of the non-fragilis isolates were ≥ 10 -fold higher than those for fully sensitive strains. The percentage of clinical isolates of *B. fragilis* (5.7%) found to possess the *cfiA* gene in the present study is substantially more than the rate reported from France (2.4%) [6], but similar to that observed by Edwards *et al.* [9].

All four *cfiA*-positive strains were obtained from patients in a large university hospital. Although these isolates would be classified as sensitive by conventional breakpoint criteria, they have the potential to convert to a high level of metallo- β -lactamase expression by acquisition of an insertion element carrying an efficient promoter of the *cfiA* gene [18].

Few clinically relevant *Bacteroides* strains have been reported world-wide to harbour the *cfiA* gene or to express metallo- β -lactamase production, resulting in carbapenem resistance and possible failure of therapy. However, Fang *et al.* [19] found the *cfiA* gene in faecal isolates of *B. fragilis* and species other than *B. fragilis* with high-level production of metallo- β -lactamase have also been isolated [20]. Careful monitoring of changes in the imipenem resistance of *Bacteroides* strains isolated from clinical samples and from normal faecal flora, together with screening for the *cfiA* gene, are important to follow the emergence and spread of carbapenem resistance in different parts of the world.

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