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Enterotoxigenic *Bacteroides fragilis* (ETBF) Strains Isolated in the Netherlands and Poland are Genetically Diverse

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A b s t r a c t

Gram-negative anaerobic rods isolated in The Netherlands and Poland from extraintestinal and intestinal sources were identified as *Bacteroides fragilis* (n = 210) on the basis of Gram staining, growth on selective Bacteroides Bile Esculine medium as black colonies, and biochemical characteristics. PCR-mediated assessment of the presence of the *B. fragilis* enterotoxin (fragilysin) gene in all strains identified 12 so-called enterotoxin-positive *B. fragilis* (ETBF) strains (15%) among the Dutch strains and 16 ETBF among the Polish strains (13%). NotI Pulsed Field Gel Electrophoresis (PFGE) analysis revealed that these strains are genetically heterogeneous. Among the Dutch strains an identical pair and a set of four indiscriminate strains were identified. This suggests that limited nosocomial spread of ETBF can be observed. However, there was no identity obeserved when strains from The Netherlands were compared to their Polish counterparts. The antimicrobial susceptibility testing revealed that one Polish strain isolated from a patient with antibiotic associated diarrhoeae (AAD) was simultaneously highly resistant to clindamycin and cefoxitin (MIC > 256 mg/L). Two other strains appeared to be clindamycin resistant. All resistant strains had different PFGE patterns, suggesting that resistance development occurred at independent occassions.

K e y w o r d s: *Bacteroides fragilis*, enterotoxin, PFGE, antibiotic resistance

Introduction

Bacteroides fragilis is a gram-negative anaerobic, asporulating, encapsulated rod. This bacterial species is bile stimulated and extremely saccharolytic. It inhabits the colon of healthy animals and humans in quantities amounting to 1% of the normal gut flora. It is the most frequently isolated anaerobe from clinical specimens. *B. fragilis* can be isolated as the etiological agent of endogenic suppurative soft tissue infections, abscesses or bacteremia (Finegold, 1995; Meisel-Mikołajczyk, 1999). Virulence factors of this bacterium were described by several autors (Hofstad 1990, 1992; Botta *et al.*, 1994; Sebald, 1996). The most important virulence factors of *B. fragilis* are, among other, its capsule, lipopolysaccharide (LPS), outer membrane protein (OMP), pili, short-chain fatty acids (Pruzzo *et al.*, 1989; Pantosti *et al.*, 1991; Poxton and Edmond, 1995; Gibson *et al.*, 1998) . The capsule influences abscess formation, antiphagocytic activity and the mode of adhesion (Sebald, 1996). The structure and biological activity of *B. fragilis* LPS was described by many authors (Meisel-Mikołajczyk and Didoszak, 1980; Mikołajczyk *et al.*, 1981; Beckmann *et al.*, 1989; Delahooke *et al.*, 1995). *B. fragilis* strains harbouring a recently described, new virulence factor called fragilysin (enterotoxin) were described by Myers and his group in 1984. The same laboratory reported on

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the isolation of these enterotoxigenic *B. fragilis* strains (ETBF) from different animals (Myers *et al.*, 1985; Myers and Shoop, 1987; Myers *et al.*, 1987a). For the first time the isolation of ETBF from humans was reported by Myers *et al.* (1987b). Very soon in different countries ETBF strains were detected in human clinical material as well. Countries including the USA (Sack *et al.*, 1992), France (Sebald and Meisel-Mikołajczyk, 1993), Italy (Pantosti *et al.*, 1994), The Netherlands (Van Belkum *et al.*, 1999), Japan (Kato *et al.*, 1995), Poland (Meisel-Mikołajczyk *et al.*, 1994; 1996a; 1996b), Bangladesh (Sack *et al.*, 1994), Sweden (Olsen *et al.*, 1999) reported on the occurrence of ETBF. As isolation of enterotoxin producing strains was described from different countries, studies on the putative relatedness of these strains have to be undertaken to determine whether or not these strains share a common source (or not).

The aim of the present study was to define whether among *B. fragilis* strains isolated in The Netherlands and Poland (from different specimens) the enterotoxin (fragilysin) producing strains can be found, whether in both countries the frequency of ETBF strains is similar and whether the strains are similar or different. This analysis is meant to shed light on strain clonality versus horizontal genetransfer as means of dissemination.

Experimental

Materials and Methods

Strains. As reference strains various well known *B. fragilis* enterotoxigenic strains were used. These included NCTC 11295 (metronidazole-resistant), ATCC 43858, and ATCC 43859. Non-ETBF included was IPL E 323. For antimicrobial susceptibility testing *B. fragilis* ATCC 25285 (non-ETBF) and *B. thetaiotaomicron* ATCC 29741were used as reference strains.

210 *B. fragilis* strains were isolated from extraintestinal and intestinal clinical samples in The Netherlands and Poland. Samples were inoculated on a BBE (Bacteroides Bile Esculin agar, BioMérieux, France) and incubated in an anaerobic chamber (Glove box, Forma Scientific Inc., USA) at 37°C for 48 hours. Identification of bacterial strains was done according to growth on selective media, colony morphology, Gram and capsule staining. API 20 A test (BioMérieux, France) was used for the biochemical identification.

PCR. DNA was isolated using the Genomic DNA PREP PLUS isolation kit manufactured by A&A Biotechnology (Gdynia, Poland).

PCR was performed in a DNA thermal cycler (Techne, UK) employing the following primer pair:

404 (5'-GAG CCG AAG ACG GTG TAT GTG ATT TGT-3') and 407 (5'-TGC TCA GCG CCC AGT ATA TGA CCT AGT-3').

PCRs were performed in incubation volumes of 25 µl containing approximately 50 ng of DNA and 0.08 U of *Taq* DNA Polymerase (Invitrogen Ltd, Paisley, United Kingdom). The cycling conditions for PCR were: 4 min at 94°C followed by 40 cycles of 1 min at 94°C, 1 min at 52°C, 1 min at 74°C. Amplification products were detected by electrophoresis in 1% agarose gel with ethidium bromide added. (Figure 1).

Only strains positive in the PCR were used in the following experiments.

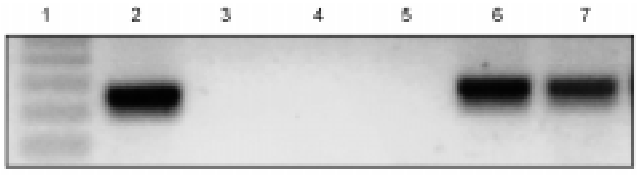

Fig. 1. Agarose gel electrophoresis of amplified DNA from selected *B. fragilis* strains.
Lane 1: 123 bp DNA ladder. Lane 2: NCTC 11295 (ETBF) strain. Lane 3: IPLE 323 (NTBF) strain Lane 4 and 5: clinical NTBF strains. Lane 6 and 7: clinical ETBF strains.

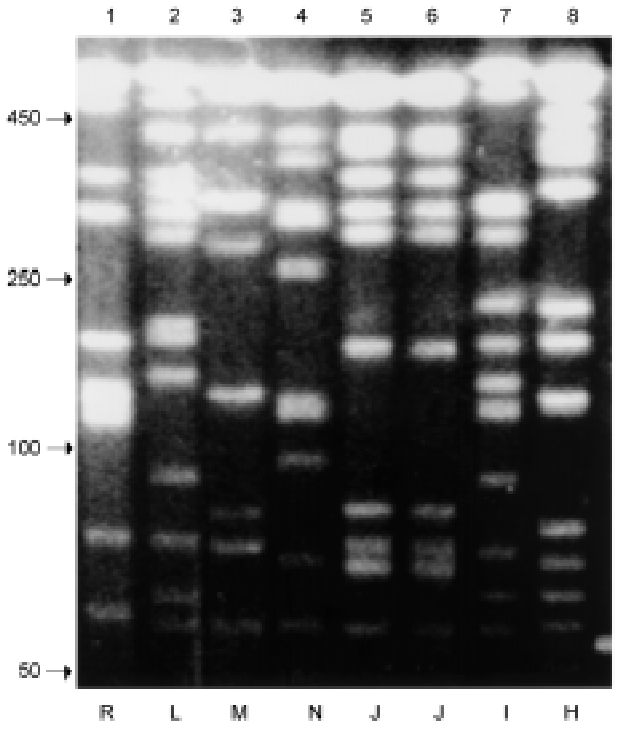

Fig. 2. Pulsed field gel electrophoresis of NotI DNA macrorestriction fragments derived from Dutch and Polish ETBF strains.
On top strain identification numbers are given. These correspond with the numbers in Table 1. 1: 2683/97; 2: 1504/2/98; 3: 1605/98; 4: 2455/98; 5: 640W; 6: 7H; 7: m1-D5; 8: 1393157. The first four strains are Polish (Warsaw), the latter Dutch (Roosendaal). On the left molecular size markers are given in kilobase pairs.

PFGE. According to Maslanka *et al.* (1999), bacteria were suspended in SE buffer (75 mM NaCl, 25 mM EDTA pH 8.0) and embedded in 0.5% agarose plugs. After solidification the plugs were immersed in lysis buffer (50 mM Tris HCl pH 8.0, 50 mM EDTA, 1% lauryl sarcosine, 1 mg/ml proteinase K). The mixture was incubated overnight at 55°C and plugs were washed 5 times in SE at room temperature afterwards. DNA in the plugs was digested using restriction endonuclease NotI (Boehringer-Mannheim, Mannheim, Germany). Electrophoresis was performed in a CHEF Mapper (BioRad, Veenendaal, The Netherlands). The voltage was 10 V/cm for 18 hours with linear sampling from 5 to 35 sek. at ± 60° angels (Figure 2).

Antimicrobial susceptibility testing of *B. fragilis* isolates. Drug susceptibility of *B. fragilis* strains was determined with Etest (AB BIODISK, Solna, Sweden) – MICs for cefoxitine, amoxicillin/clavulanic acid, imipenem, clindamycin and metronidazole. MICs were estimated in accordance to the NCCLS recommendations (1997).

Results and Discussion

Among the *B. fragilis* strains, 12/78 strains (15%) from The Netherlands and 16/132 Polish strains (13%) contained the fragilysin gene. The percentage of ETBF strains isolated in The Netherlands (strains isolated from extraintestinal materials) and Poland (strains isolated mainly from fecal samples of patients with AAD) is very similar. The PFGE analysis (Figure 2, Table I) revealed that these strains are genetically hetrogeneous.

Table I
Origin of the *B. fragilis* ETBF strains and PFGE results

No.	Strain	Country	Material	PFGE
1.	074	The Netherlands[1]	Extraintestinal	E
2.	075		Extraintestinal	E
3.	2938723		Smear Douglas abscess	E
4.	086		Extraintestinal	F
5.	3187323		Blood	G
6.	1393157		Fluid ascites	H
7.	m1-D5		Extraintestinal	I
8.	7H		Extraintestinal	J
9.	640W		Extraintestinal	J
10.	14H		Extraintestinal	*
11.	29		Clinical specimens	AA
12.	7		Punctate of femur	AB
13.	W1	Poland[2]	Child feces (without diarrhoea)	AC
14.	W2		Child feces (with diarrhoea)	AD
15.	P51		Fecal sample (AAD)	K
16.	P131		Fecal sample (AAD)	AG
17.	2683/97		Fecal sample (AAD)	R
18.	1504/2/98		Fecal sample (AAD)	L
19.	1605/98		Fecal sample (AAD)	M
20.	2455/98		Fecal sample (AAD)	N
21.	2465/3/98		Fecal sample (AAD)	*
22.	2785/98		Fecal sample (AAD)	O
23.	210/2/99		Fecal sample (AAD)	P
24.	1502/99		Fecal sample (AAD)	Q
25.	2/B		Pus	*
26.	76/D		Oral cavity (child)	AF
27.	26 CD/2000		AAD infant feces	AH
28.	III		Pus	AI
29.	NCTC 11295	Reference strains		AE
30.	ATCC 43858			B
31.	ATCC43859			A
32.	IPL E 323 (NTBF)			D

[1] The Netherlands
Franciscusziekenhuis Medical Mikrobiology, Roosendaal. Strains nos 1–10, Department of Medical Microbiology and Infectious Diseases, Erasmus University Medical Center, Rotterdam. Strains nos 11 and 12.
[2] Poland
Department of Medical Microbiology, The Medical University of Warsaw, Warsaw. Strains nos 13–26, Department of Bacteriology, Hospital, Płock. Strain no 27, Danuta Dzierżanowska, Department of Microbiology and Immunobiology, The Children's Memorial Health Institute, Warsaw. Strain no 28.
* These strains are probably very rich in endogenous DNase, and maybe it can be a separate biotype.

Table II
Susceptibility to antimicrobial agents of *Bacteroides fragilis* ETBF strains (MIC mg/l)

No	Strain	Cefoxitin (FX)	Amoxi./Clav. (XL)	Imipenem (IP)	Clindamycin (CM)	Metronidazole (MZ)
1.	074	8	0.25	0.047	1.0	0.19
2.	075	12	0.25	0.032	0.75	0.19
3.	2938723	8	0.125	0.023	1.0	0.19
4.	086	4	0.125	0.023	1.0	0.38
5.	3187323	8	0.25	0.064	1.5	0.50
6.	1393157	8	0.19	0.064	0.25	0.19
7.	m1-D5	4	0.125	0.032	0.125	0.38
8.	7 H	4	1.0	0.125	0.023	0.25
9.	640W	6	1.0	0.19	0.032	0.19
10.	14H	6	1.0	0.19	0.75	0.38
11.	29	4	1.5	0.25	0.094	0.125
12.	7	4	0.125	0.032	0.25	0.125
13.	W1	16	0.125	0.032	0.125	0.25
14.	W2	4	0.125	0.023	0.5	0.125
15.	P51	12	0.25	0.032	1.5	0.38
16.	P131	>256	2	1.5	>256	0.19
17.	2683/97	4	0.25	0.064	0.032	0.38
18.	1504/2/98	16	0.5	0.25	>256	0.38
19.	1605/98	8	0.19	0.032	0.75	0.38
20.	2455/98	4	0.5	0.5	0.094	0.38
21.	2465/3/98	6	0.5	0.5	0.19	0.50
22.	2785/98	4	0.38	0.125	0.125	0.19
23.	210/2/99	4	0.25	0.047	0.50	0.50
24.	1502/99	8	0.19	0.047	0.75	0.25
25.	2/B	16	1.0	0.125	>256	1.5
26.	76/D	6	0.19	0.047	0.25	1.5
27.	26 CD/2000	6	0.5	0.125	0.125	0.5
28.	III	8	0.5	0.094	<0.016	0.125
29.	NCTC 11295	4	1.5	0.064	<0.016	>32
30.	IPLE 323 (NTBF)	6	0.094	0.023	0.125	0.125
31.	ATCC 25298	4	0.38	0.032	0.5	0.25
32.	BT ATCC 29741[1]	8	0.5	0.064	2.0	0.5

[1] BT – *Bacteroides. thetaiotaomicron*.
Breakpoints MIC (mg/l) in accordance to NCCLS (1997): cefoxitin – 64, amoxicillin/clavulanic acid – 16, imipenem – 16, clindamycin – 8, metronidazole – 32.

Three strains (no 14H, 2465/3/98 and 2/B) are probably very rich in endogenous DNase, and maybe it represents a separate biotype. Interestingly, type E was encountered three times among the Roosendaal strains, whereas type J was seen for two strains. This suggests the nosocomial spread. Strains from the two different countries did not reveal any identity among each other.

All tested strains (Table II) were susceptible to amoxicillin/clavulanic acid (MIC 0,125–2 mg/L), imipenem (MIC 0,023–1,5 mg/L) and metronidazole (MIC 0,125–1,5 mg/L). Two tested strains (nos 1504/2/98 and 2/B) were highly resistant to clindamycin. One strain (no P131 isolated from patient with AAD) was simultaneously highly resistant to clindamycin and cefoxitin (MIC > 256 mg/L). Remaining strains were sensitive to clindamycin (MIC < 0,016–1,5 mg/L) and cefoxitin (MIC 4–16 mg/L).

From the presented studies two general conclusions can be drown:

1. It is very important that all enterotoxigenic *Bacteroides fragilis* strains observed in this study are genetically nonhomogenous.
2. The most active (*in vitro*) antibacterials against ETBF strains under investigations are amoxicillin with clavulanic acid, imipenem and metronidazole.

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