PCR ribotyping and arbitrarily primed PCR for the comparison of enterotoxigenic *Bacteroides fragilis* strains from two Polish university hospitals

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Objective: To study the clinical incidence and possible clonal relatedness of enterotoxigenic strains of *Bacteroides fragilis* among pediatric and adult patients in two Polish university hospitals.

Methods: Fecal samples from 201 adults and 131 infants (with or without diarrhea) and vaginal samples from 100 pregnant women nursed in two Polish university hospitals were analyzed with respect to carriage of enterotoxin-producing *Bacteroides fragilis* (ETBF). This putative pathogen was identified by cultivation and subsequent cyto-pathogenicity testing of culture supernatants on HT/29 C1 cells.

Results and discussion: Two ETBF strains were isolated from childrens' feces; two additional strains were isolated from adults, and from the vaginal samples only a single strain was isolated. One strain (W2) was isolated from a child with diarrhea. These incidence figures, the fact that all ETBF isolates were shown to produce strongly differing amounts of the cytotoxin, and the genetic unrelatedness of the strains as demonstrated by two different PCR-mediated DNA typing procedures, indicates that clonal spread of ETBF is presently not a clinical problem in these hospitals. It was shown that PCR-mediated ribotyping and arbitrarily primed PCR can be applied with success to study the epidemiology of ETBF.

Key words: PCR ribotyping, arbitrarily primed PCR, *Bacteroides fragilis*, molecular typing, enterotoxin

INTRODUCTION

Bacteroides fragilis is an anaerobic bacterial species forming part of the normal human colonic flora [1,2]. On the other hand, *B. fragilis* is commonly isolated from human clinical specimens, and the bacterium is known to cause intra-abdominal abscesses and blood-stream infections [3–5]. However, neither diarrheal disease nor enterotoxin production due to *B. fragilis* was appreciated until 1984. The first report concerning a *B. fragilis* toxin was written by the veterinary research team of Myers [6]. This report identified for the first time an enterotoxigenic activity associated with *B. fragilis* which was reported to cause diarrhea in lambs. Since that initial publication many cases of *B. fragilis* enterotoxin-induced diarrhea in calves, piglets and foals have been reported [7–9]. Moreover, enterotoxigenic *B. fragilis* (ETBF) has recently been associated with watery diarrheal disease in livestock and young children. Joaquin et al. [10] suggested that some of the indigenous *B. fragilis* produce enterotoxin constitutively. This toxin has been shown to have two major biological activities: stimulation of water secretion in ligated intestinal segments of lambs and calves, and alteration of the morphology of intestinal epithelial cell lines in vitro [11]. Consequently, ETBF strains can now be detected by an in vitro cytotoxicity assay with HT-29 cells [12]. Recently, we showed that in Poland individuals with or without diarrhea can harbor ETBF strains in the intestine [13]. The present

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knowledge concerning the spread and incidence of ETBF among hospitalized patients is limited, and detailed epidemiologic studies are clearly needed.

Several molecular epidemiologic typing procedures that can be applied to many microbial species have been developed [14,15]. Recently, the arbitrarily primed polymerase chain reaction (AP PCR) and PCR ribotyping have been employed successfully as well [16–19]. In theory, most of these procedures can be successfully applied to *B. fragilis* and should be useful for the elucidation of interstrain relationships among isolates of ETBF. The aim of the present study was to genetically characterize ETBF strains isolated in Warsaw (Poland) and compare them with reference ETBF strains by epidemiologic studies and PCR-mediated genotyping.

MATERIALS AND METHODS

Reference ETBF strains

B. fragilis ATCC 43858, ATCC 43859 (both isolated from human feces), 86-5443-2-2 (from pig feces), 3-101-5 (from pig feces), and NCTC 11295 (from human feces) [20] were used as reference strains in this study.

Clinical material

Fecal samples were obtained from patients with or without diarrhea (201 adults and 131 children) housed in the no. 1 and no. 8 Warsaw university hospitals. Not all patients were screened, but once a diarrheic patient was identified, his or her room-mates were included in the study as well. Vaginal samples were obtained from 100 pregnant healthy women. Samples were collected in a non-systematic, randomized fashion during the period between 1992 and 1996. The current collection of ETBF (see also below) may be considered a cross-sectional, representative group of isolates.

Cultivation and identification of ETBF

Bacteroides Bile Esculin (BBE) agar and Columbia blood agar with kanamycin and vancomycin were used for the cultivation of *B. fragilis* strains from the fecal samples. Identification of *B. fragilis* was based on a commercially available method (API-20A, bioMerieux, France). Four to five different colonies were subjected to biochemical testing; only one was selected for examination of cytotoxicity. To assess enterotoxin production, each *B. fragilis* strain was grown overnight in brain–heart infusion broth (BHI) supplemented with hemin (5 μg/mL) at 37°C. The cultures were centrifuged and the supernatants were concentrated approximately 20-fold. The supernatants were frozen immediately and kept at −20°C until use [21,22]. Cytotoxicity assays on HT/29C1 cells were performed as described previously [13]. Briefly, HT/29C1 cells from one flask were resuspended in 20 mL of medium, distributed (200 μL/well) into a 96-well microtiter plate and allowed to grow for 2 to 3 days until discrete clusters of cells were visible. Medium was removed, 180 μL of fresh medium was added to each well, and 20-μL aliquots of two-fold dilutions of bacterial culture supernatants were then inoculated into the wells in duplicate. The plate was incubated at 37°C in air with 5% CO_2 and examined after 2 and 4 h for the presence of the typical toxin-induced cytopathic changes. The culture supernatants were considered to contain *B. fragilis* enterotoxin if a cytopathic effect was visible which could be neutralized by a neutralizing antibody from a rabbit antitoxic antiserum [13]. The cytotoxic titer was the highest dilution of the culture supernatant that affected at least 50% of the cells after incubation for 4 h, an effect neutralized by the antiserum.

DNA isolation

The protocol used for DNA isolation has been described in detail elsewhere [e.g. 17]. In short, *B. fragilis* was cultured for 24 to 48 h in BHI. The culture was centrifuged at 3000*g* for 10 min and the resulting bacterial pellet was processed for DNA isolation using lysozyme and the chaotropic salt guanidine isothiocyanate. The specific binding of DNA to Celite was employed for affinity purification [23,24]. The DNA concentration in the remaining eluate was estimated by electrophoresis of small aliquots in parallel with samples with a known DNA concentration.

AP PCR

AP PCR was performed as described previously [17]. Primers were the enterobacterial repetitive intergenic consensus sequences ERIC1 and ERIC2, used either separately or in combination [25]. The nucleotide sequences of these primers are 5′-CACTTAGGGG TCCTCGAATGTA-3′ and 5′-AAGTAAGTGACT GGGGTGAGCG-3′. One additional primer (AP7) was used to corroborate the findings obtained with the ERIC primers. The sequence of AP7 is 5′-GTGG ATGCGA-3′. A 40-cycle program was applied (1 min at 94°C + 1 min at 25°C + 2 min at 74°C). Banding patterns were compared visually and any different fingerprint was indicated with a capital letter. Even single band differences were indicative for the establishment of a novel type.

PCR ribotyping

Analyses of the variation in length of the ribosomal intergenic spacer regions was performed as described elsewhere [17]. Use of primers SP1

(5′-TTGTACACACACCGCCCGTCA-3′, specific for the 16S rRNA gene) and SP2 (5′-GGTACCTTAGATGTTTCAGTTC-3′, specific for the 23S rRNA gene) in combination with a PCR program consisting of a 40 cycles of alternating denaturation (1 min at 94°C), primer annealing (1 min at 55°C) and primer extension (1 min at 74°C) led to visualization of the amplified stretches of DNA after gel electrophoresis. Amplified DNA was analyzed by electrophoresis in 1% agarose gels run in × 0.5 TBE at 100 mA constant current.

Scanning and Gelcompar analysis of PCR fingerprints

The pictures as generated by PCR ribotyping and AP PCR were scanned by use of a Hewlett Packard Scanjet IIc document scanner. TIF files were introduced in the Gelcompar software (Applied Maths, Leuven, Belgium) and data were converted and normalized visually. The degree of banding pattern homology was determined by Dice comparisons of the peaks, and clustering correlation coefficients were calculated by the unweighed pair-group method with arithmetic averages (UPGMA).

RESULTS

Epidemiologic surveillance

From the childrens' feces 45 strains of Gram-negative, anaerobic rods were isolated. Seven of them proved to be *B. fragilis*. A single ETBF strain (W1) was isolated from fecal samples of infants without diarrhea (n=80). From children with diarrhea (n=51), another ETBF strain (W2) was cultivated. The clinical data for the ETBF-colonized children were not different from those of the other children. Only non-diarrheic adults were included in the present survey; *B. fragilis* was never cultured from the feces of diarrheic patients. This rendered an overall number of 131 isolates of Gram-negative, anaerobic rods, of which 29 represented *B. fragilis*. Two ETBF strains (no. 32 and no. 154) were isolated from adults without diarrhea. Only one ETBF strain (no. 131) was isolated from the vagina of a single pregnant woman. Altogether, five out of 432 persons (1.1%) were demonstrated to be carriers of ETBF. The heterogeneity of the ETBF strains was already indicated by the variability in cytotoxin titers observed (from 10 to 160). Three additional non-enterotoxigenic *B. fragilis* strains (192, 190, 180), isolated from fecal samples of infants without diarrhea, were used in this study.

PCR-mediated genotyping

The results of AP PCR and PCR ribotyping are shown partly in Figure 1 and the data interpretation is summarized in Table 1. From Figure 1 it can be deduced that the results obtained with primer AP7 are largely corroborated by the data obtained with primers ERIC1 and ERIC2. The differences between the banding patterns are quite subtle. In lane 1 (Figure 1B), for instance, a unique DNA fragment is visible just below the 200-bp marker molecule. Lanes 1 and 2 differ also by a single versus a double band between the 800- and 900-bp markers. Lane 3 displays different spacing between precisely those two DNA fragments. Lane 4 harbors an additional band in the just over 200-bp region, which sets the fingerprint pattern apart from the one in lane 2. The PCR ribotyping may show differing grouping results but most of the data are concordant with the AP PCR results. As seen in

Table 1 Survey of the typing data of different enterotoxigenic *B. fragilis* strains

No.	Strain number	Source of strains	HT-29 CA cytotoxin titer	AP PCR				Ribo spacer	Overall type
				E1	E2	E1/E2	AP7		
1.	131	Vagina	160	A	A	A	A	1	1
2.	192	Infant	NT	B	B	B	B	2	2
3.	190	Infant	NT	A	A	C	C	3	3
4.	154	Adult	10	C	C	D	D	4	4
5.	32	Adult	80	B	A	E	E	2	5
6.	W2	Infant	160	D	ND	D	F	5	6
7.	W1	Infant	160	E	D	F	G	6	7
8.	NCTC11295	Human	160	F	A	D	H	7	8
9.	3-101-5	Pig	80	G	E	E	I	1	9
10.	86-5443-2-2	Pig	320	H	F	G	G	8	10
11.	ATCC43859	Human	160	I	G	H	J	8	11
12.	ATCC43858	Human	320	ND	D	G	G	8	12
13.	180	Infant	NT	I	C	I	K	9	13

NT = non-toxigenic *B. fragilis* strains; ND = not detected. Overall type = combination of data obtained with both PCR ribotyping and AP PCR.

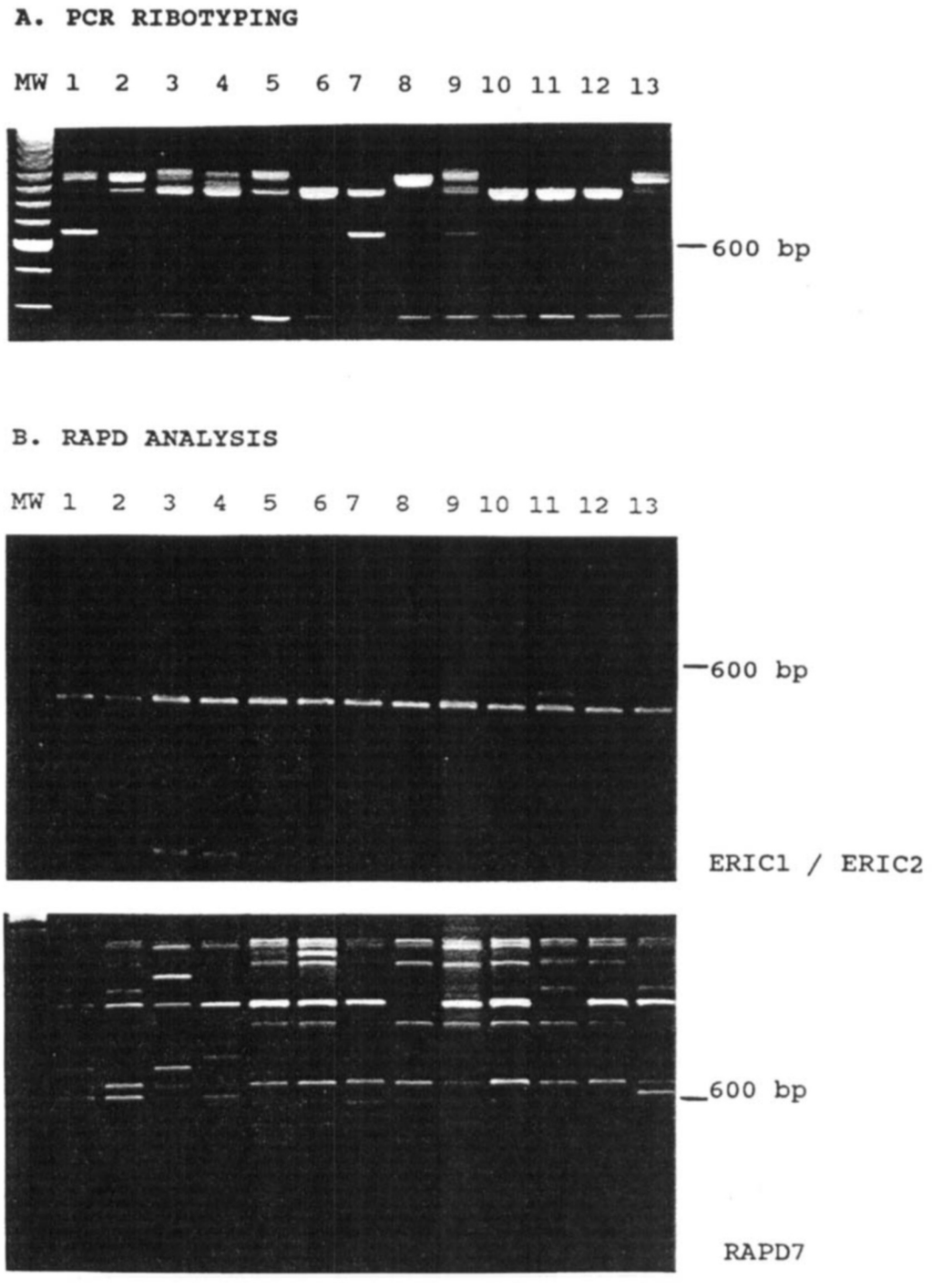

Figure 1 Survey of experimental data obtained with PCR-mediated typing of the ETBF strains. (A) PCR-mediated ribotyping of the clinical ETBF isolates and the reference strains. The numbering above the lanes corresponds with numbering as presented in Table 1. On the left a molecular length marker was co-electrophoresed (100-bp ladder, Pharmacia, Woerden, the Netherlands); the most intensely staining band has a length of 600 bp. (B) AP PCR for the same panel of strains. The length marker is identical to the one described above. Only the results of the ERIC1–ERIC2 combination assay and the tests with RAPD7 are displayed.

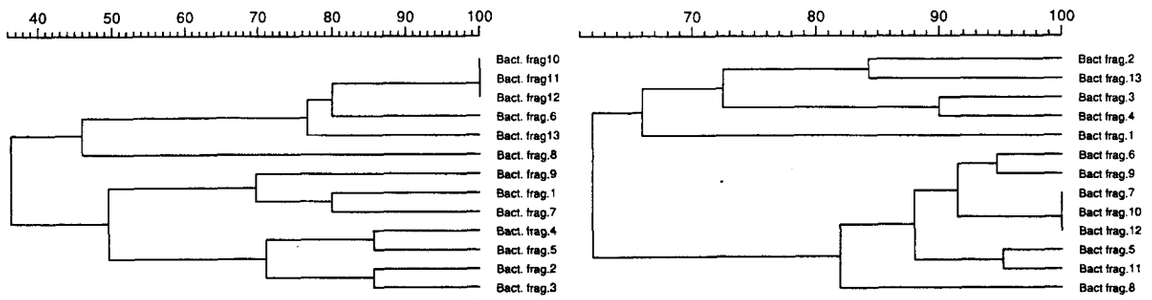

Figure 2 Dendrograms generated for the PCR ribotyping data and the AP PCR fingerprints generated with primer RAPD7 (see also Figure 1). The degree of banding pattern homology was determined by Dice comparisons, and clustering correlation coefficients of the peaks were calculated by the unweighed pair-group method with arithmetic averages (UPGMA). The numbering of the strains corresponds with those mentioned in Table 1. The scale bar indicates the percentage similarity among fingerprints. The panel on the left shows the interpretation of the ribotyping results. On the right the data obtained by AP PCR with RAPD7 are highlighted.

Table 1, all of the clinical isolates can be easily differentiated from the reference strains and the non-ETBF. When all data are gathered in a single digit (column 'Overall type' in Table 1), all of the 13 isolates included in the present comparison are genetically unique. When the data are digitized and phylogenetic trees are constructed (Figure 2), the heterogeneity that was already established by the visual examination is confirmed. Only a limited set of strains display genetic homology giving rise to cluster formation in the UPGMA analysis. Strains that cluster with respect to, for instance, the PCR ribotyping (strains 10, 11 and 12) can only be partially separated by AP PCR tests. Based on the automated analysis it can be once more concluded that all strains were unrelated except strains 10 and 12. Since these strains are isolated from the feces of completely non-related organisms like the pig and human being, respectively, it is suggested that the larger PCR-detected differences observed among the other strains are genuine determinants of genetic distance. In order to confirm possible transmission of ETBF from an animal reservoir to human individuals, additional studies are required.

DISCUSSION AND CONCLUSIONS

Part of the gene encoding the *B. fragilis* enterotoxin was recently cloned by single specific primer PCR [26]. The enterotoxin appeared to be a metalloprotease capable of lysing a large number of proteins involved in the maintenance of cell morphology. Since the enterotoxigenic activity is clearly disease-linked, studies into the spread and incidence of ETBF are urgently required. Anticipation of possible rises in occurrence and insights into the mode of transmission of ETBF may be helpful for the design of preventive measures. For this reason a surveillance study was performed among patients nursed in two Warsaw university hospitals, and PCR-mediated methodology was applied to assess clonality among the strains encountered in this particular clinical setting. Application of novel typing strategies for elucidation of ETBF epidemiology is required, since serotyping of ETBF does not adequately differentiate strain variants and, thus, may not provide sufficient discriminatory power for typing of these strains [27]. Moreover, serotyping does not discriminate non-enterotoxigenic *B. fragilis* from ETBF. Since the application of the PCR-mediated procedures appeared to be successful for the epidemiologic study of *Clostridium difficile* [17], in this study different *B. fragilis* strains were compared by the same PCR typing methods. Similarity between ETBF strains isolated in Poland [28,29] and reference ETBF strains was not observed, and nor was there any genetic linkage among the clinical isolates themselves, leading to the conclusion that there is no evidence from this study that spread of ETBF strains that present a clinical problem is taking place as yet. Nevertheless, among some reference ETBF strains some resemblances in ribotype and AP PCR type were observed. The validity of AP PCR for typing of *B. fragilis* is confirmed by a recent study on clonality among strains possessing the metallo-beta-lactamase gene *cfiA* [30]. It was demonstrated that grouping obtained with conventional ribotyping was concordant with the results of AP PCR. Furthermore, the resolution of AP PCR appeared to be primer

dependent, but, generally, more types were identified by AP PCR than by ribotyping using ribosomal DNA probes. In general, reliable typing of ETBF can be performed successfully by any of the techniques mentioned above. However, we feel that as long as no inter-institutional consensus agreement exists on the optimal ETBF typing protocol, results obtained by a single technique should by verified with an alternative approach. Awaiting further studies, we postulate that single band differences detected by the AP PCR studies described in this communication really define separate ETBF types. This is underscored by the fact that PCR ribotyping of strains 1 and 2 (see Figure 1A) reveals gross differences, reminiscent of relatively large genetic distances, while the AP PCR fingerprints show single band differences only.

In conclusion, it can be stated that clonal spread of ETBF among hospitalized patients in two of the major hospitals in Warsaw (Poland) is not in progress. The ETBF incidence is low, and those strains that are encountered are clearly polyclonal in nature. Larger studies in geographically more diverse regions are currently underway.

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