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Discrimination of *Burkholderia multivorans* and *Burkholderia vietnamiensis* from *Burkholderia cepacia* Genomovars I, III, and IV by PCR

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We present a PCR procedure for identification of *Burkholderia cepacia*, *Burkholderia multivorans*, and *Burkholderia vietnamiensis*. 16S and 23S ribosomal DNAs (rDNAs) of *B. multivorans* and *B. vietnamiensis* were sequenced and aligned with published sequences for definition of species-specific 18-mer oligonucleotide primers. Specific antisense 16S rDNA primers (for *B. cepacia*, 5′-AGC ACT CCC RCC TCT CAG-3′; for *B. multivorans*, 5′-AGC ACT CCC GAA TCT CTT-3′) and 23S rDNA primers (for *B. vietnamiensis*, 5′-TCC TAC CAT GCG TGC AA-3′) were paired with a general sense primer of 16S rDNAs (5′-AGR GTT YGA TYM TGG CTC AG-3′) or with a sense primer of 23S rDNA (5′-CCT TTG GGT CAT CCT GGA-3′). PCR with these primers under optimized conditions is appropriate to specifically and rapidly identify *B. multivorans*, *B. vietnamiensis*, and *B. cepacia* (genomovars I, III, and IV are not discriminated). In comparison with the polyphasic taxonomic analyses presently necessary for species and genomovar identification within the *B. cepacia* complex, our procedure is more rapid and easier to perform and may contribute to clarifying the clinical significance of individual members of the complex in cystic fibrosis.

The spectrum of pathogens isolated from respiratory specimens of cystic fibrosis (CF) patients has changed within the last 60 years (1). The prevalence of *Pseudomonas aeruginosa* has increased, in contrast with that of *Staphylococcus aureus* (18). Furthermore, new species have emerged, e.g., *Burkholderia* (*Pseudomonas*) *cepacia*, which was first detected in CF patients in about 1971 (20). The prevalence of *B. cepacia* was 7.1% among 283 patients at CF centers in Munich, Germany, between 1987 and 1997 (4) and 5.6% among 180 patients of the CF centers at the University Clinic in Essen, Germany, between 1993 and 1998 (8). Govan et al. (15) reported a prevalence of 7% in Scotland in 1992. An average prevalence of 3.6% was reported for patients from 113 Cystic Fibrosis Foundation-accredited centers in the United States by S. FitzSimmons (11a).

Since 1984 (20), it has been known that CF patients who acquire *B. cepacia* may follow one of three different clinical courses: persistent colonization without additional deterioration of lung function, significantly accelerated reduction of lung function, or—in about 20% of the patients—rapid deterioration with signs and symptoms of acute necrotic pneumonia with or without septicemia and fatal outcome within several weeks or months ("cepacia syndrome" [20]). The factors which determine this dissociation may be either pathogen- or patient-related, or both.

Provided that the incoming *Burkholderia* strain strongly determines the further course of CF lung infection, the strains isolated from patients who develop the "cepacia syndrome" may be different from strains of CF patients without rapid clinical decline. So far, all *Burkholderia* isolates collected from CF patients were uniformly identified by conventional methods to be *B. cepacia* and did not appear to be heterogeneous. Thus, it was not possible to correlate the heterogeneity in the clinical course with a specific *Burkholderia* subgroup. This is, however, of major importance for the individual patient and for the community of persons with CF.

In fact, Vandamme et al. (31) reidentified 128 isolates conventionally characterized as *B. cepacia* by DNA-DNA and DNA-ribosomal DNA (rDNA) hybridization, assimilation of organic carbon sources, whole-cell protein pattern, and fatty acid profile. They subclassified the strains into five species, namely *B. vietnamiensis* (genomovar V), *B. multivorans* (genomovar II), and the *B. cepacia* genomovars I, III, and IV (these have no names, as their identification by phenotypic markers is inconclusive so far). Strains of *B. vietnamiensis* (14), *B. multivorans* (8), and the *B. cepacia* genomovars I, III, and IV (31) have been detected in respiratory specimens of CF patients. This subclassification of strains previously identified as *B. cepacia* stimulated the advancement of the hypothesis that the nature of the incoming *Burkholderia* strain determines or strongly influences the clinical course of CF lung disease. It has been difficult to evaluate this hypothesis because appropriate procedures for reliable and rapid identification of the *Burkholderia* species and genomovars have not been available. There are, however, reports suggesting that *B. cepacia* genomovar III strains were predominantly associated with outbreaks in North America and Europe (24a, 33). This underlines the importance of accurate genomovar identification.

Currently, the *B. cepacia* genomovars I, III, and IV, *B. multivorans*, and *B. vietnamiensis* can be discriminated by the procedures used for their description (see above). These procedures are not suitable for routine diagnostic work. Instead, a number of biochemical reactions were proposed for phenotypic differentiation (9, 14, 21, 31); however, not all strains can be reliably identified, e.g., nonsaccharolytic strains (for example, biovar C of *B. cepacia* genomovar III), and the evaluation of the tests may take 3 to 5 days for metabolically deficient, slow-growing strains. We therefore tried to find a procedure for specific identification of *Burkholderia* spp. based on the

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diversity within the nucleotide sequences of their 16S and 23S rDNAs. Sequence motifs characteristic for single species were integrated into oligonucleotide primers. They allow the production of PCR products specific for *B. multivorans*, *B. vietnamiensis*, and the *B. cepacia* genomovars I, III, and IV. Thus, PCR-based molecular procedures to discriminate the *Burkholderia* species relevant in CF, including *Burkholderia gladioli* (6), are now available.

MATERIALS AND METHODS

Organisms. For characterization of the strains used in this study, see Table 1.

Nucleic acid preparation. Genomic DNA was prepared and purified by using the QiaAmp purification kit (Qiagen, Hilden, Germany).

PCR. (i) For sequencing of the PCR products. Custom oligonucleotide primers (see Table 2) were purchased from MWG Biotech, Ebersberg, Germany. Amplification reactions were carried out in a 50-μl final volume with 1 U of proofreading *Taq* polymerase provided with the Expand High Fidelity PCR System (Boehringer, Mannheim, Germany), 5 μl of the reaction buffer supplied by the manufacturer (diluted 1:10), 10 μmol of each deoxynucleotide triphosphate, and 50 pmol of each oligonucleotide primer. Approximately 50 to 100 ng of DNA was used as a template. The PCR was carried out in a GeneAmp PCR system 9600 (Applied Biosystems, Weiterstadt, Germany) under the following conditions: denaturation for 5 min at 95°C, followed by 30 amplification cycles of 30 s at 95°C, 30 s at the annealing temperature specific for the primer, and 45 s at 72°C. The samples were then incubated at 72°C for 7 additional min and cooled to 4°C. Pyrogen-free water that had been shown to be free of contaminating DNA was used throughout the study. To inactivate contaminating DNA, the PCR mixture was exposed to UV light for 15 min prior to addition of enzyme and template. To ascertain reproducibility, all species identifications by PCR were performed in duplicate, starting each time from new cultures. The amplification products were checked by agarose gel electrophoresis and purified of salt and excess primers by using the PCR purification kit (Qiagen).

(ii) For identification of unknown strains. The same procedure as described above was followed, with some exceptions: half of the volumes of all amplifications reagents was used, and normal *Taq* polymerase was used instead of the proofreading *Taq* of the High Fidelity PCR System. For each reaction, a specific antisense primer was combined with the universal sense primer Eub-16-1, and for identification of *B. vietnamiensis* the primers ViMaPs-23-1 and CeVi-23-2 were used (Table 3).

Agarose gel electrophoresis. Agarose gel electrophoresis was performed as described previously (5).

Sequence determination. The amplified rDNAs were sequenced as described previously (23). The two strands of the DNA were sequenced from different PCR products. A model 373A DNA Sequencer (Applied Biosystems) was used, following the protocol of the manufacturer for dye terminator reactions.

Analysis of the sequence data. The nucleotide sequences were aligned with reference rDNA sequences provided in the noncommercial software program package ARB (beta-version 2.4). Secondary structure analysis was done according to the method of Ludwig et al. (24).

Nucleotide sequence accession numbers. The 16S and 23S rDNA sequences of *B. multivorans* (*B. cepacia* genomovar II) LMG 13010^T and the 23S rDNA sequence of *B. vietnamiensis* (*B. cepacia* genomovar V) LMG 10929^T have been deposited in the EMBL database under the accession nos. 418703, 418704, and 418705.

TABLE 1. Investigated strains

Organism	Strain designation or origin[a]
B. vietnamiensis	LMG 10929^T, LMG 6998, LMG 6999, CF isolate[b]
B. cepacia	DSM 50181, ATCC 25416^T, LMG 7000, LMG 12614, LMG 12615, LMG 14294, CF isolates[c]
B. multivorans	LMG 14280, LMG 14293, CF isolates[d]
B. gladioli	DSM 4285, CF isolates[e]
B. mallei	ATCC 23344^T, ATCC 15310, ATCC 10399
B. pseudomallei	ATCC 23343^T, ATCC 15682, NCTC 1691, clinical isolates[f]
R. pickettii	ATCC 27511^T, clinical isolate[g]

[a] DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Federal Republic of Germany; LMG, Laboratorium voor Microbiologie, Universiteit Gent, Gent, Belgium; ATCC, American Type Culture Collection, Rockville, Md.; NCTC, National Collection of Type Cultures, Central Public Health Laboratory, London, England.
[b] Strain from The Netherlands ($n = 1$).
[c] Strains from Germany ($n = 34$), The Netherlands ($n = 2$), and the United Kingdom ($n = 6$).
[d] Strains from Germany ($n = 60$), The Netherlands ($n = 11$), and Denmark ($n = 8$).
[e] Strains from Germany ($n = 2$) and Canada ($n = 3$).
[f] Strains from Germany ($n = 1$) and Switzerland ($n = 1$).
[g] Strain from Germany ($n = 1$).

RESULTS

Analysis of 16S and 23S rDNA sequences. 16S rDNA sequences of *B. cepacia* were found in the literature (24). We sequenced 16S rDNA of reference strains (Table 1) of *B. multivorans* and *B. vietnamiensis* and of the *B. cepacia* genomovars I and III kindly supplied to us by P. A. R. Vandamme (Gent, Belgium). Multiple alignment of the sequences of various strains demonstrated among the species substitutions of nucleotides included within the variable helix 18 or 37/38 regions. They were used for the definition of species-specific primers (Table 2). No sequences appropriate for discrimination between *B. multivorans* and *B. vietnamiensis* were detectable in the 16S rDNA. However, analysis of the 23S rDNA revealed sites with nucleotides that were different from those in the other *Burkholderia* spp. included but were identical in the four *B. vietnamiensis* strains investigated. Thus, as shown for differentiation of *Burkholderia mallei* and *Burkholderia pseudomallei* (5), the 23S rDNA sequences may be useful when no suitable nucleotide substitutions are detectable in the 16S rDNA genes.

Specificity of PCR. Primers were combined to form pairs and were used to establish optimal conditions for specificity. Each pair of opposite primers was analyzed for the specificity in comparison with those of all other *Burkholderia* species or genomovars and of *Ralstonia pickettii*. No amplification products were detected with heterologous *Burkholderia* strains (Table 3). Furthermore, we studied representative strains of species which grow on tryptone soya agar supplemented with 16 μg of colistin/ml. Two strains each of the following species were included: *Proteus mirabilis*, *Morganella morganii*, *Serratia liquefaciens*, *Alcaligenes xylosoxidans*, *Stenotrophomonas maltophilia*, *Brevundimonas diminuta*, and *P. aeruginosa* (MIC of colistin, >64 μg/ml). In addition, strains assumed to be *Burkholderia* spp. were sent to us for verification. Some of them were negative according to our PCR procedure, so they were subjected to conventional identification reactions. They proved to be *A. xylosoxidans* ($n = 15$), *P. aeruginosa* ($n = 10$), *S. maltophilia* ($n = 6$), *M. morganii* ($n = 3$), and *Citrobacter freundii* ($n = 3$). The results were definitive, and no amplification products obtained with any of the specific primer pairs shown in Table 3 were detectable with templates of these strains.

Procedure for identification of *Burkholderia* spp. For identification of strains shown to be *Burkholderia* spp. by API 20NE, four separate PCRs are run. They included primers of different degrees of specificity in order to achieve stepwise exclusion of single species: Bu-Ra excludes species of genera other than *Burkholderia* or *Ralstonia*, CeMuVi-16-2_{457} excludes *B. gladioli* and *Ralstonia* spp., MuVi-16-2_{1028} excludes *B. cepacia*, and the 23S rDNA primers specific for *B. vietnamiensis* exclude *B. multivorans*. Except for *B. cepacia*, which includes the three genomovars I, III, and IV, all species can be identified by positive reactions (*B. vietnamiensis*, *B. gladioli*, and *R. pickettii*) or exclusion (*B. multivorans*). For the *B. cepacia* genomovars I, III, and IV, no specific signatures were found within 16S or 23S rDNA sequences. Strains yielding products of PCR with the Bu-Ra primers but negative for PCR with the other primers may be either *B. mallei* or *B. pseudomallei*. They can be identified as described previously (5).

TABLE 2. Primers selected for PCR

Target species	Primer designation[a]	Sequence[b]	Annealing temp (°C)
Eubacteria	Eub-16-1	5′-AGR GTT YGA TYM TGG CTC AG-3′	56
Eubacteria	Eub-16-2	5′-ACC GCG GCT GCT GGC AC-3′	60
Burkholderia spp. and *Ralstonia* spp.	BuRa-16-1	5′-AGC ACT TTT GTC CGG RAA-3′	52
Burkholderia spp. and *Ralstonia* spp.	BuRa-16-2	5′-AAT CCC CAA CAA CTA GTT G-3′	54
B. cepacia, *B. multivorans*, and *B. vietnamiensis*	CeMuVi-16-2_{457}	5′-CCG RCT GTA TTA GAG CCA-3′	55
B. gladioli	Gl-16-2_{457}	5′-CGA AGG ATA TTA GCC CTC-3′	54
B. cepacia	Ce-16-2_{1028}	5′-AGC ACT CCC RCC TCT CAG-3′[c]	59
B. multivorans and *B. vietnamiensis*	MuVi-16-2_{1028}	5′-AGC ACT CCC GAA TCT CTT-3′	54
B. mallei and *B. pseudomallei*	MaPs-16-2_{457}	5′-CTC CGG GTA TTA GCC AGA-3′	56
Ralstonia pickettii	Pi-16-2_{461}	5′-TCG ACC CCA GGT ATT AAC-3′	54
B. vietnamiensis, *B. mallei*, and *B. pseudomallei*	ViMaPs-23-1	5′-CCT TTG GGT CAT CCT GGA-3′	56
B. cepacia and *B. vietnamiensis*	CeVi-23-2	5′-TCC TAC CAT GCG TGC AA-3′	52
B. mallei	Ma-23-2	5′-CAC CGA AAC TAG CA-3′	42

[a] 16, 16S rDNA; 23, 23S rDNA; 1, sense (forward) primer; and 2, antisense (backward) primer.
[b] Wobble bases: M = A or C; R = A or G; and Y = C or T.
[c] Partially congruent with primer PSR1 of Campbell et al. (11), i.e., 5′-TTTCGAGCACTCCCGCCTCTCAG-3′.

Evaluation of the primers with CF isolates. We studied 124 isolates collected from CF patients from four European countries. They had been identified by conventional phenotypic means as *B. cepacia* and collected for PCR. All of the strains could be classified. Of the isolates 79 (63.7%) were found to be *B. multivorans*, 42 (33.9%) were *B. cepacia*, 2 (1.6%) were *B. gladioli* (Hannover, Germany), and 1 (0.8%) was *B. vietnamiensis* (Amsterdam, The Netherlands). *B. multivorans* appears to be the dominating species among *Burkholderia* isolates from European CF centers investigated in this work (7). No major spread of *B. multivorans* strains was observed among 10 patients of one CF center. Altogether, eight different randomly amplified polymorphic DNA types were identified, and only two of them were detected in more than one patient (8).

DISCUSSION

In CF, the basic genetic defect predisposes the individual to lung infections caused by specific bacterial pathogens. The major gram-negative organisms causing chronic recurrent lung inflammation are *P. aeruginosa* and *Burkholderia* spp. Once established, these pathogens can be eradicated only infrequently and transiently. Nevertheless, the increase in life expectancy and quality of life achieved within the last decade is mainly attributed to progress in antiinfective therapy. Therefore, even transient reduction in the concentration of pathogens at the site of infection benefits the clinical status and prognosis of the patient. As long as gene therapy is not available antimicrobial treatment must be optimized, along with measures for the prevention of early infection and the transmission of pathogens among CF patients.

These measures rely on the knowledge of the microbiological status of the CF patient at all times. Exact data on the identity of the pathogens and their concentrations in respiratory secretions (mostly sputum) as well as on their susceptibility to therapeutically relevant antibiotics allow the rational design of treatment regimens and effective strategies for controlling the spread of these pathogens from infected to pathogen-free patients.

At this time, the genus *Burkholderia* is thought to be composed of 16 species (10, 32, 36). Most of these are primarily plant pathogens; the CF-relevant *Burkholderia* species are restricted to *B. cepacia* (genomovars I, III, and IV), *B. multivorans*, *B. gladioli*, and *B. vietnamiensis*. Their detection in diagnostic laboratories depends initially on the use of selective media which inhibit growth of *P. aeruginosa*. All *Burkholderia* spp. of potential relevance in CF lung disease grow on colistin-supplemented media (12, 17, 19, 34). In a quality control trial carried out by Tablan et al. (30), only 32% of the participating

TABLE 3. Specificity of primer combinations for the identification of *Burkholderia* spp.

Primer designation[a]	Target organisms	Size of PCR product (bp) for:								
		B. cepacia genomovar			*B. multivorans*	*B. vietnamiensis*	*B. gladioli*	*B. mallei*	*B. pseudomallei*	*R. pickettii*
		I	III	IV						
Eub-16-1 + Eub-16-2	*Eubacteria*	519	519	519	519	519	519	519	519	519
BuRa-16-1 + BuRa-16-2	*Burkholderia* and *Ralstonia* spp.	409	409	409	409	409	409	409	409	409
Eub-16-1 + CeMuVi-16-2_{457}	*B. cepacia*, *B. multivorans*, and *B. vietnamiensis*	463	463	463	463	463				
Eub-16-1 + Ce-16-2_{1028}	*B. cepacia*	1,015	1,015	1,015						
Eub-16-1 + MuVi-16-2_{1028}	*B. multivorans* and *B. vietnamiensis*				1,015	1,015				
ViMaPs-23-1 + CeVi-23-2	*B. vietnamiensis*					1,055				
Eub-16-1 + Gl-16-2_{457}	*B. gladioli*						463			
Eub-16-1 + MaPs-16-2_{457}	*B. mallei* and *B. pseudomallei*							463	463	
ViMaPs-23-1 + Ma-23-2	*B. mallei*							526		
Eub-16-1 + Pi-16-2_{461}	*R. pickettii*									467

[a] For sequences see Table 2.

115 laboratories detected *B. cepacia* in the test sputum. In a surveillance study including 78 centers in Germany conducted in 1997, the prevalence of *Burkholderia* spp. was 4.1% when a selective medium containing colistin was used but only 1.8% when selective medium was not used (our own unpublished data). In fact, only about one-fifth of the sputa were analyzed by using a *Burkholderia*-selective agar. On colistin-containing media, besides *Burkholderia* spp., a variety of other species are able to grow (e.g., *Proteus* spp., *Providencia* spp., *M. morganii*, *Serratia* spp., *A. xylosoxidans*, and staphylococci).

For identification of *Burkholderia* spp., the procedures used for their taxonomic description (13, 28) (see Introduction) are inappropriate in clinical laboratories. The aim of our work was to establish procedures which meet the demands of clinical practice where determination of both the identity of a pathogen and its antibiotic susceptibility is called for. For this purpose, culturing of the organisms is indispensable. Thus, our identification procedure is based on culture. The procedure may also be adapted to detect *Burkholderia* spp. in situ, e.g., in sputum specimens. The major advantage of the PCR-based identification is rapidity. While evaluation of the biochemical reactions proposed for phenotypic differentiation (9, 14, 21, 31) may take up to 7 days (17), the PCR procedure is performed within 5 h. The biochemical tests for reliable identification are not commercially available now and have to be prepared and controlled for quality within individual laboratories. They are inappropriate for the identification of nonsaccharolytic strains (e.g., biovar C of *B. cepacia* genomovar III [31]).

Molecular identification of pathogens relies on the diversity of the nucleotide sequences of the 16S and 23S rDNA sequences with specific motifs conserved at different phylogenetic levels, e.g., genus and species. This diversity has been used for species-specific PCR procedures (22, 26, 28). Our 16S and 23S rDNA sequence data for *B. vietnamiensis*, *B. multivorans*, and the *B. cepacia* genomovars I, III, and IV demonstrated options for discriminatory primers. Among them, the oligonucleotides listed in Table 2 were found to be appropriate for the identification of *B. multivorans*, *B. vietnamiensis*, and the *B. cepacia* genomovars I, III, and IV, which could not be separated until now. The different PCRs may be run separately to discriminate simultaneously *B. cepacia* (genomovars I, III and IV are included), *B. multivorans*, or *B. vietnamiensis* from colonies identified conventionally as a *Burkholderia* species. A mixture from different colonies (instead of a pure culture) should be tested to find out whether the specimen contains more than one species.

A molecular identification procedure for *B. cepacia* that uses 16S rDNA sequences was proposed by Campbell et al. (11). This work preceded the changes of taxonomy in 1997 (31) and therefore could not include efforts to differentiate the five *B. cepacia* complex genomovars. Analysis of the primers described by Campbell et al. demonstrated specificity for *B. cepacia*. No PCR products were amplified from DNA templates of *B. multivorans* and *B. vietnamiensis* (our own unpublished data), while discrimination of *B. gladioli* had already been shown by the authors. Whitby et al. (35), in addition to the primers described by Campbell et al. (11), used a primer pair, G-1 and G-2, targeting the 130-bp spacer region between 16S and 23S rDNA. However, the spacer-directed primers were less specific than the 16S-rDNA-derived primers. The PCR procedures which we present facilitate rapid and specific identification of *B. multivorans*, *B. vietnamiensis*, *B. gladioli* (6), and *B. cepacia*. However, the primers proposed for identification of *B. cepacia* do not discriminate among the *B. cepacia* genomovars I, III, and IV. Work for molecular differentiation of these genomospecies is in progress.

The basic question has been asked whether members within a genus of mostly phytopathogenic species can be primary causative pathogens in CF or whether they are just indicators of a late stage of lung disease. The pros and cons have been presented and discussed by different authors (13, 16). It is so far uncertain whether among the *Burkholderia* spp. in CF patients, there are species with increased virulence (25) which on their own or in combination with cofactors (e.g., preceding damage of lung tissue by other microorganisms) cause the rapid clinical deterioration of the patient. Major outbreaks in Canada and the United Kingdom were caused by the spread of particular strains which produce cblA protein (27, 29) and appear to be *B. cepacia* genomovar III. This may indicate that virulence might be associated with a particular type or species of pathogen and that accurate identification of the species of the *B. cepacia* complex is of major clinical importance. The PCR-based procedures proposed to discriminate *B. cepacia*, *B. multivorans*, *B. vietnamiensis*, and *B. gladioli* should help to clarify their prevalence, epidemiology, antibiotic susceptibility, and pathogenetic potential in CF (2, 3).

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