Identification of *Burkholderia* spp. in the Clinical Microbiology Laboratory: Comparison of Conventional and Molecular Methods

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Cystic fibrosis (CF) predisposes patients to bacterial colonization and infection of the lower airways. Several species belonging to the genus *Burkholderia* are potential CF-related pathogens, but microbiological identification may be complicated. This situation is not in the least due to the poorly defined taxonomic status of these bacteria, and further validation of the available diagnostic assays is required. A total of 114 geographically diverse bacterial isolates, previously identified in reference laboratories as *Burkholderia cepacia* (*n* = 51), *B. gladioli* (*n* = 14), *Ralstonia pickettii* (*n* = 6), *B. multivorans* (*n* = 2), *Stenotrophomonas maltophilia* (*n* = 3), and *Pseudomonas aeruginosa* (*n* = 11), were collected from environmental, clinical, and reference sources. In addition, 27 clinical isolates putatively identified as *Burkholderia* spp. were recovered from the sputum of Dutch CF patients. All isolates were used to evaluate the accuracy of two selective growth media, four systems for biochemical identification (API 20NE, Vitek GNI, Vitek NFC, and MicroScan), and three different PCR-based assays. The PCR assays amplify different parts of the ribosomal DNA operon, either alone or in combination with cleavage by various restriction enzymes (PCR-restriction fragment length polymorphism [RFLP] analysis). The best system for the biochemical identification of *B. cepacia* appeared to be the API 20NE test. None of the biochemical assays successfully grouped the *B. gladioli* strains. The PCR-RFLP method appeared to be the optimal method for accurate nucleic acid-mediated identification of the different *Burkholderia* spp. With this method, *B. gladioli* was also reliably classified in a separate group. For the laboratory diagnosis of *B. cepacia*, we recommend parallel cultures on blood agar medium and selective agar plates. Further identification of colonies with a *Burkholderia* phenotype should be performed with the API 20NE test. For final confirmation of species identities, PCR amplification of the small-subunit rRNA gene followed by RFLP analysis with various enzymes is recommended.

Pulmonary colonization with *Burkholderia cepacia* is associated with a poor clinical prognosis for patients with cystic fibrosis (CF) (23). A relatively constant fraction of CF patients in Western European countries appear to be colonized with this usually multiple-antibiotic-resistant organism. However, the prevalence in individual CF centers may differ widely due to epidemic bursts of infection and problems with the identification of the microorganism. Interestingly, in some CF patients, long-term colonization can occur without an adverse effect on lung function (8). On the other hand, some individuals deteriorate rapidly after colonization, and death may occur within 1 to 6 months (8). There is also evidence that particular clonal isolates of *B. cepacia* can be easily transmitted from person to person (12, 25, 31, 32). Separating *B. cepacia*-colonized or -infected patients from other CF patients has been used to prevent bacterial spread, but this practice has a severe social and psychological impact on the patients and

their family members (10). In The Netherlands, colonization with *B. cepacia* is considered a contraindication for lung transplantation. However, this point of view is not internationally acknowledged (19). Because of the serious implications of the identification of *B. cepacia* in patients with CF, microbiological diagnosis should be carried out as accurately as possible. However, the isolation and reliable identification of *B. cepacia* have been complicated and difficult (6, 9, 15, 21). Although the pathogenicity of the closely related species *B. gladioli* has not been definitely assessed, reliable pathogenicity studies can only be performed once precise species identification can be achieved in the routine microbiology laboratory.

B. cepacia has recently been described as a complex of multiple genetic types, or genomovars: *B. cepacia* (genomovar I), *B. multivorans* (genomovar II), genomovars III and IV (36), and the closely related new species *B. vietnamiensis* (formerly genomovar IV) (4, 9, 33). Strains of all of these genomovars, *B. vietnamiensis*, and *B. gladioli* have been isolated from CF patients. Several of the *Burkholderia* spp. are of commercial importance because they can be used as biopesticides, as plant growth promoters, or for the degradation of environmental pollutants (4, 18, 22, 33).

In The Netherlands, the identification of putative *B. cepacia*

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TABLE 1. Geographical, clinical, and microbiological characteristics of the strain collection[a]

Origin	Organism	No. of isolates	Source	Method of identification by the study participants
RIVM[b]	B. cepacia	2	Sputa, lung biopsy	16S rRNA sequence
	B. gladioli	5	CF patient	Biochemical
	S. maltophilia	3	Sputa	
University of British Columbia, Vancouver, British Columbia, Canada	B. cepacia	4	CF patient	API 20NE, biochemical (15, 23)
		1	Soil (ATCC 25416)	
	B. multivorans	1	CF patient	
		1	Soil (ATTC 17616)	
	B. gladioli	2	CF patient, CGD	
		1	Onion (ATCC 10248)	
Sweden	B. cepacia	4	CCUG 788, CCUG 12691, CCUG 9631, CCUG 36978	
	R. pickettii	2	CCUG 3314, CCUG 3318	
	B. gladioli	2	CCUG 1782, CCUG 2115	
Medizinische Hochschule, Hannover, Germany	B. cepacia	33	CF patient	*Spe*I fragment pattern and/or sequencing of 16S rRNA, BCSA, OFPBL, API 20NE
		1	Environment	
	B. gladioli	2	CF patient	
Universite Paul Sabatier, Toulouse, France	B. cepacia	3	ATCC 25416, ATCC 17759, ATCC 25609	
	B. gladioli	2	ATCC 10248, ATCC 19302	
AZR; SKZ[c]	B. cepacia	2	ATCC 25416, ATCC 17759	
		1	SKMM	
	P. aeruginosa[f]	11	ATCC CF patient	MicroScan and PC480-PC1250 PCR
	R. pickettii	4	CF patient Environment	MicroScan and API 20NE, BCSA
	Unspecified	19	CF patient	PC480-PC1250 PCR
LVF[d]	Unspecified	2	Patient	
		2	CF patient	Biochemical
AZU[e]	Unspecified	4	CF patient	API 20NE

[a] For *B. cepacia*, 10 reference strains, 2 environmental isolates, and 37 clinical strains were included in the present analysis. For *B. gladioli*, these figures are 4, 1, and 4, respectively. One clinical isolate and one environmental isolate of *B. multivorans* are included. For *P. aeruginosa*, 11 reference and clinical strains were studied. Two *R. pickettii* reference strains and four clinical isolates of this species were analyzed. Finally, 25 "unidentified" bacterial isolates were included. The *Spe*I fragment patterns were generated by pulsed-field gel electrophoresis of DNA macrorestriction fragments. SKMM, Foundation for Quality Control in Medical Microbiology, Groningen, The Netherlands; ATCC, American Type Culture Collection, Manassas, Va.; CCUG, Culture Collection of the University of Goteborg, Goteborg, Sweden; CGD, chronic granulomatous disease.
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[e] University Hospital Utrecht, Utrecht, The Netherlands.
[f] *P. aeruginosa* strains were not isolated from CF patients and did not have a mucoid phenotype.

isolates is generally performed by conventional biochemical analyses. Different institutions use different approaches; even when essentially the same diagnostic scheme has been applied, several discrepancies have become apparent upon close evaluation and comparison of the diagnostic data obtained. Moreover, the lack of a well-evaluated and generally accepted diagnostic "gold standard" technique is considered a significant limitation by all of those involved in the management of and care for CF patients. In this communication, we address the lack of gold standard microbiology techniques and describe the results of conventional and molecular identification procedures applied to a large and diverse collection of *Burkholderia* strains. Finally, a method for the optimal identification of

B. cepacia and other clinically relevant *Burkholderia* spp. is suggested on the basis of the results of this comparative study.

MATERIALS AND METHODS

Collection and initial identification of strains. Several strains of *B. cepacia*, *B. gladioli*, *Ralstonia pickettii* (formerly *B. pickettii* [39]), *B. multivorans*, *Stenotrophomonas maltophilia*, and *Pseudomonas aeruginosa* were obtained from expert microbiology centers in Germany, Canada, France, Sweden, and The Netherlands. The characteristics of the strains are given in Table 1. Note that some of the reference strains obtained from the different participants are the same. *B. cepacia* ATCC 25416, for instance, was obtained from three centers. The strain used in our Rotterdam laboratory was obtained directly from the American Type Culture Collection and, as such, provided an accurate species control specimen.

Table 1 summarizes the methods of identification used in the strain contributor home laboratories.

All strains were characterized by a combination of microbiological, biochemical, and molecular methods at a single, central laboratory (Department of Medical Microbiology and Infectious Diseases, Erasmus University Medical Centre Rotterdam EMCR, Rotterdam, The Netherlands). All individual assays were performed batchwise by a single researcher to prevent experimental day-to-day and person-to-person variability.

Presumptive identification with selective culture media. All strains were subcultured on *B. cepacia* selective agar (BCSA) (15) and oxidation-fermentation base-polymyxin B-bacitracin-lactose medium (OFPBL; Becton-Dickinson, Heidelberg, Germany) (38). The plates were incubated for 5 days at 30 or 37°C. Growth on BCSA is considered indicative of *B. cepacia*. Colonies on OFPBL were suspected of being *B. cepacia* if they were yellow as a result of lactose oxidation in the presence of the bromothymol blue indicator. The natural yellow pigment of bacterial strains other than *B. cepacia* may lead to misidentification and a false-positive result, however.

Biochemical testing. All bacterial isolates were characterized on the basis of phenotypic characteristics by use of four commercial assays. Prior to inoculation into these systems, a subculture was made on a brucella blood agar plate (bioMerieux, Marcy l'Etoile, France), which was incubated at 37°C for 18 h. Oxidase production was determined by means of a dipstick oxidase test (Difco, Detroit, Mich.).

API 20NE. For all strains, a cell suspension having a 0.5 McFarland optical density (MF) standard was made with 0.85% NaCl. Appropriate amounts of this material were added to the wells of an API 20NE strip (bioMerieux), some of which were covered with mineral oil. The strips were incubated for 48 h at 30°C, and the results were recorded by visual inspection and scored with an APILAB PLUS software package as proposed by the manufacturer.

Vitek analysis. For identification, two different Vitek cards (from bioMerieux at location given above and at s'-Hertogenbosch, The Netherlands) were used. One card is routinely used for the identification of gram-negative bacteria (GNI), and the other card has been developed for the industrial identification of non-fermenters (NFC). The latter is not commercially available yet. For all strains, a suspension having a 1.0 MF standard was made with 0.45% NaCl. The cards, filled with the suspensions, were placed in a specific tray, which was placed in the Vitek combined reader-incubator. Identification with the NFC was performed by use of the equipment of the Dutch bioMerieux representative in s'-Hertogenbosch, The Netherlands.

MicroScan analysis. For identification by use of MicroScan technology (Dade International, West Sacramento, Calif.), the so-called "negative urine combo type 1" was used. This test format also assays the susceptibility of strains to antibiotics frequently used against urinary tract infections and/or infections caused by gram-negative bacteria. A cell suspension having a 0.5 MF standard was made with 0.45% NaCl. One hundred microliters of this suspension was pipetted into a tube with water containing pluronic acid to avoid air bubbling. The MicroScan kit was filled with the cell suspension and placed in a WalkAway reader-incubator for overnight processing.

PCR analyses. PCR-mediated identification was performed by means of three methods directed to various parts of the ribosomal gene operon (26, 29, 35, 37). Prior to DNA isolation, strains were grown overnight at 37°C on brucella blood agar plates. One to three colonies were suspended in 25 mM Tris-HCl (pH 8.0)–10 mM EDTA–50 mM glucose and treated with proteinase K and 10% sodium dodecyl sulfate (SDS). DNA was purified by affinity chromatography with guanidine hydrothiocyanate and Celite (Janssen Pharmaceuticals, Beerse, Belgium) (5). The DNA concentration was estimated by electrophoresis with 1% agarose gels (Hispanagar; Sphaero Q, Leiden, The Netherlands) in the presence of known quantities of lambda DNA as references. The PCR mixtures used for all amplifications contained 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 2.5 mM MgCl$_2$, 0.01% gelatin, 0.1% Triton X-100, 0.2 mM respective deoxyribonucleotide triphosphate, 2 U of *Taq* polymerase (Sphaero Q), and 50 pmol of each primer. Amplification of DNA was performed with a model 60 thermocycler (Biomed, Theres, Germany). Amplicons were analyzed by electrophoresis with 1% agarose gels in the presence of a 100-bp DNA ladder for size assessment (Gibco/BRL Life Technologies, Breda, The Netherlands).

Two diagnostic PCR assays were performed; the first was done with primers PC1 and PC2 (35). This assay amplifies the ribosomal internal transcribed spacer (ITS) spanning the distance between the 16S and the 23S ribosomal genes. Approximately 300 ng of DNA was added to a PCR mixture. Amplification involved an initial denaturation of 2 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 68°C, and 1 min at 72°C. A final extension of 30 min at 72°C was performed. The target sequence comprised 323 bp. In the second diagnostic PCR assay, primers PC480 and PC1250 were used (26). This amplification reaction, targeting the 16S ribosomal gene, should result in the specific detection of *B. cepacia*. Approximately 150 ng of DNA was added to a standard PCR mixture. Amplification was performed according to the following scheme: initial denaturation of 2 min at 96°C, followed by 25 cycles of 15 s at 96°C, 30 s at 52°C, and 1.5 min at 70°C. A final extension of 5 min at 70°C was included. The target sequence was 770 bp long.

PCR-RFLP. Amplification of the 16S rRNA gene, followed by restriction enzyme-mediated fragmentation of the amplicon (restriction fragment length polymorphism [RFLP] analysis), should result in *Burkholderia* sp.-specific band-

TABLE 2. Growth of *Burkholderia* spp. and other gram-negative bacilli on two selective media

Organism[a]	No. of isolates	No. (%) growing on:	
		BCSA	OFPBL
B. cepacia	50	48 (96)	50 (100)
B. gladioli	14	6 (43)	8 (57)
R. pickettii	2	2 (100)	0 (0)
B. multivorans	2	2 (100)	2 (100)
S. maltophilia	3	0 (0)	1 (33)
P. aeruginosa	11	0 (0)	0 (0)

[a] Identification as provided by the different strain contributors.

ing patterns (29, 37). Approximately 50 ng of DNA was added to a PCR mixture as described above. PCR was performed with primers rD1 and fD1 and a 35-cycle program of 2 min at 95°C, 30 s at 42°C, and 4 min at 72°C, with a final extension of 20 min at 72°C. The amplicons, approximately 1,500 bp long, were analyzed and quantified on a 1% agarose gel (Hispanagar). After amplification, the samples were treated with four different restriction enzymes (*Alu*I, *Cfo*I, *Msp*I, and *Dde*I; Boehringer GmbH, Mannheim, Germany). These restriction enzymes were selected on the basis of homology searches performed for *Burkholderia* sp.-specific 16S rRNA sequences available through GenBank (see Fig. 2). One microliter of enzyme mixture containing 1 U of restriction enzyme and the appropriate buffer components was added to 9 μl of the amplified product. The mixture was incubated for 2 h at 37°C. The sizes of the restriction fragments were documented by electrophoresis, ethidium bromide staining, UV transillumination, and photography.

RESULTS

Culture-based assays. All of the *B. cepacia* and *B. gladioli* strains were positive in the oxidase assay. The results of growth analyses with selective agar plates are shown in Table 2. Forty-eight of 50 strains of *B. cepacia* grew on both BCSA and OFPBL plates. The two isolates not growing on BCSA plates were gentamicin-sensitive *B. cepacia*, a result which implies that gentamicin susceptibility may not be a 100% reliable species discriminator. These isolates were derived from a single German CF patient, showed identical random amplified polymorphic DNA patterns, were negative for the *B. cepacia* epidemicity marker, and belonged to the *B. cepacia* genomovar III group (16).

Biochemical identification. It must be mentioned explicitly that the taxons *B. gladioli* and *B. multivorans* are not included in the databases of any of the assays used here. The results of biochemical assays are shown in Table 3. Overall, the API 20NE and the Vitek GNI were the most accurate. The API 20NE gave a doubtful but essentially correct identification ("low level of discrimination") for 6% of the strains; for only 2% of the strains could the system not provide a bacterial identification at all. The Vitek GNI could not identify 10% of the tested strains. None of the systems was able to identify *B. gladioli* correctly and efficiently. The API 20NE identified 36% of these strains as *B. cepacia*. The Vitek GNI identified 21% of *B. gladioli* strains as *B. cepacia*, whereas the Vitek NFC and the MicroScan scored 50% and none, respectively, of 14 strains of *B. gladioli* as *B. cepacia*. The MicroScan identified 43% of the strains as nonfermenters, and for 57% of the strains an incorrect identification was given. The two strains from Canada which were presented as *B. multivorans* were identified as *B. cepacia* by all four systems. All systems could reliably identify two strains of *R. pickettii*, the *P. aeruginosa* strains, and two strains of *S. maltophilia*. The Vitek NFC approach did not correctly identify one of the *S. maltophilia* strains tested.

PCR assays. The results shown in Table 4 and Figure 1 summarize the experimental data obtained with the PCR-RFLP procedure. The diagnostic PCRs with primers PC480-

TABLE 3. Comparison of four systems of biochemical identification for *Burkholderia* spp. and various other bacterial species[a]

Organism	No. of isolates	System	No. (%) of isolates with the following identification:			
			Correct[b]	Incomplete	Incorrect	Unidentified
B. cepacia	50	Vitek GNI	45 (90)	0 (0)	0 (0)	5 (10)
		Vitek NFC	34 (68)	0 (0)	11 (22)	5 (10)
		API 20NE	45 (90)	3 (6)	1 (2)	1 (2)
		MicroScan	34 (68)	0 (0)	16 (32)	0 (0)
B. gladioli	14	Vitek GNI	0 (0)	0 (0)	6 (43)	8 (57)
		Vitek NFC	0 (0)	0 (0)	9 (64)	5 (56)
		API 20NE	0 (0)	0 (0)	14 (100)	0 (0)
		MicroScan	0 (0)	0 (0)	14 (100)	0 (0)
R. pickettii	6	Vitek GNI	6 (100)	0 (0)	0 (0)	0 (0)
		Vitek NFC	4 (100)	0 (0)	0 (0)	0 (0)
		API 20NE	4 (66)	1 (17)	0 (0)	1 (17)
		MicroScan	6 (100)	0 (0)	0 (0)	0 (0)
B. multivorans	2	Vitek GNI	2 (100)	0 (0)	0 (0)	0 (0)
		Vitek NFC	2 (100)	0 (0)	0 (0)	0 (0)
		API 20NE	2 (100)	0 (0)	0 (0)	0 (0)
		MicroScan	2 (100)	0 (0)	0 (0)	0 (0)
S. maltophilia	3	Vitek GNI	3 (100)	0 (0)	0 (0)	0 (0)
		Vitek NFC	2 (66)	0 (0)	0 (0)	1 (33)
		API 20NE	3 (100)	0 (0)	0 (0)	0 (0)
		MicroScan	3 (100)	0 (0)	0 (0)	0 (0)
P. aeruginosa	11	Vitek GNI	11 (100)	0 (0)	0 (0)	0 (0)
		Vitek NFC	10 (91)	0 (0)	0 (0)	1 (9)
		API 20NE	11 (100)	0 (0)	0 (0)	0 (0)
		MicroScan	11 (100)	0 (0)	0 (0)	0 (0)
Unspecified clinical isolates[c]						
B. cepacia	20	Vitek GNI	9 (45)	5 (25)	1 (5)	5 (25)
		Vitek NFC	8 (40)	0 (0)	0 (0)	12 (60)
		API 20NE	16 (80)	3 (15)	1 (5)	0 (0)
		MicroScan	5 (25)	0 (0)	15 (75)	0 (0)
P. aeruginosa	5	Vitek GNI	1 (20)	2 (40)	2 (40)	0 (0)
		Vitek NFC	2 (40)	0 (0)	0 (0)	3 (60)
		API 20NE	4 (80)	0 (0)	1 (20)	0 (0)
		MicroScan	0 (0)	0 (0)	5 (100)	0 (0)

[a] Note that *B. gladioli* is not mentioned in the databases of the systems.
[b] Identification to the species level with a probability of more than 80%.
[c] For the evaluation of this group of isolates, PCR-RFLP was considered the gold standard for identification.

PC1250 and PC1-PC2 could not accurately differentiate *B. cepacia* from *B. gladioli* (Table 4). The PCR with the PC1-PC2 primer combination, whose major drawback is that it lacks sensitivity for *B. cepacia*, provided a positive result for only 22 of 50 *B. cepacia* strains (sensitivity, 44%). The specificity of this PCR was good, since no positive results were encountered among the different *B. gladioli* strains. Although the PC480-PC1250 PCR correctly recognized all strains from both species (100% sensitivity), it did not distinguish between *B. cepacia* and *B. gladioli*. Moreover, other species may produce positive signals as well (e.g., *Alcaligenes* spp. [unpublished observation]), rendering this diagnostic application useless.

RFLP typing of ribosomal amplicons turned out to be the most appropriate technique for discriminating *B. cepacia* from *B. gladioli*. All *B. gladioli* strains were found identical when *Alu*I and *Dde*I digests were considered (type BC). For *B. cepacia*, the *Alu*I pattern was characteristic (type A). These assays were diagnostically accurate and corroborated the initial laboratory identification. On the other hand, the RFLP anal-

yses demonstrated intraspecies heterogeneity (types AAAA, AAAB, and ABBB for *B. cepacia*, for instance). Interestingly, several of these single RFLP patterns were shared by the two species (A and B patterns for *Msp*I, for instance). The RFLP patterns obtained for the other species clearly differed from the *B. cepacia* and *B. gladioli* patterns (e.g., types DDDE, EEFH, and CCCD). Clearly, strains identified as *B. cepacia* were genetically heterogeneous, as were *P. aeruginosa* strains (types EEFH, FEFH, and GFFH). It is interesting to note that 19 of the *B. cepacia* strains generated a type AAAA PCR-RFLP pattern, which is identical to that recorded for the *B. multivorans* strains. This finding is in agreement with the recent proposals of Vandamme et al. (36), who separated *B. multivorans* from the bulk group of *B. cepacia* as a separate type. Type AAAA is also encountered among clinical isolates, substantiating the fact that isolates belonging to this specific genomovar have colonizing and/or pathogenic potential.

Analysis of clinical *Burkholderia* sp. isolates. Twenty of 27 (74%) of the clinical isolates that were suspected of being

TABLE 4. Results of PCR-mediated identification

| Organism[a] | No. of strains | No. (%) identified with the following primer pair: | | PCR-RFLP results[d] | |
		PC1-PC2[b]	PC480-PC1250[c]	Type	No. (%) of strains with the indicated type
B. cepacia	50	22 (44)	50 (100)	AAAB	20 (40)
				AAAA	19 (38)
				ABBB	11 (22)
B. gladioli	14	0	14 (100)	BBBC	10 (71)
				BBEC	4 (29)
R. pickettii	6	0	0	DDDE	2 (100)
B. multivorans	2	0	2 (100)	AAAA	2 (100)
S. maltophilia	3	0	0	CCCD	3 (100)
P. aeruginosa	11	0	0	EEFH	3 (27)
				FEFH	6 (55)
				GFFH	2 (18)
Clinical isolate[e]	27	2 (7)	21 (78)	AAAA	18 (67)
				AAAB	2 (7)
				EEFH	5 (19)
				Other	2 (7)

[a] Identification as provided by the strain contributors.

[b] Increased level of discordance for *B. cepacia* compared to results with PC1250-PC480, but correctly negative for the other species.

[c] Apparently suited for identifying both *Burkholderia* species; however, non-*Burkholderia* species also may be identified.

[d] Order of restriction enzymes used: *Alu*I, *Cfo*I, *Msp*I, and *Dde*I.

[e] RFLP was considered the gold standard for identification.

B. cepacia appeared to be genuine *B. cepacia* when PCR-RFLP was considered the gold standard for identification. This finding implies that the different Dutch strain contributors possibly misidentified at least seven strains, five of which were identified as *P. aeruginosa* by RFLP. This figure is even higher when the other forms of identification are considered. Based on Vitek NFC analysis, for instance, 12 of 20 *B. cepacia* strains (60%) would not have been identified correctly. It must be emphasized here that the Vitek NFC has not yet been qualified for laboratory-based medical microbiological diagnostic procedures. The API 20NE misidentified only a single strain, underscoring its excellent diagnostic potential. Not all strain contributors misidentified the strains; since most of the strains were isolated from CF patients and showed aberrant characteristics, the microbiologists involved wanted to exclude the possibility of *B. cepacia* carriage.

DISCUSSION

The genetic defect causing CF predisposes patients to an aberrant pulmonary susceptibility to infectious disease. Several of the bacterial species encountering a suitable ecological niche in the lungs of CF patients are especially pathogenic for the host. *B. cepacia*, a microorganism originally identified as the causative agent of soft rot of onions, is a well-known representative of this particular group; in 20% of patients who become colonized with this species, the rapidly fatal *B. cepacia* syndrome may occur (11). Microbiological detection of *B. cepacia* appears to be complicated, and laboratory proficiency
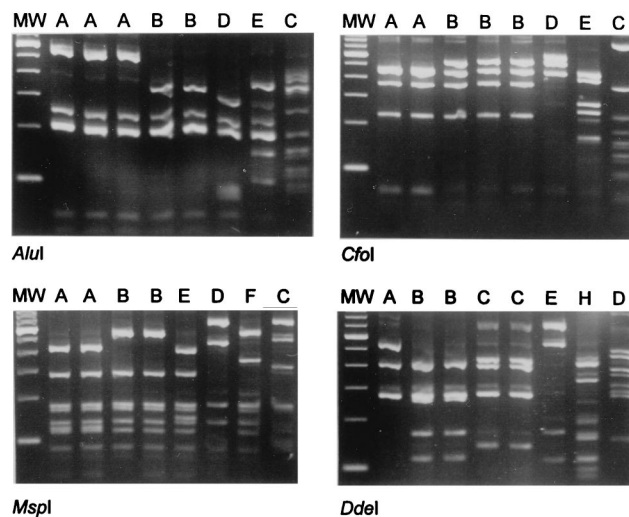

FIG. 1. PCR-RFLP analysis of *Burkholderia* spp. and other gram-negative bacilli. The four panels display the results obtained by restriction of small-subunit rRNA amplicons with the restriction endonucleases indicated below the panels. The lanes show the RFLP types found in each enzyme assay. DNA templates were derived from the following strains (from left to right) (RFLP type): *B. cepacia* Dutch patient isolate (AAAA), *B. cepacia* ATCC 25416 (AAAB), *B. cepacia* H134-6 (ABBB), *B. gladioli* ATCC 10248 (BBBC), *B. gladioli* RIVM 95-665 (BBEC), *R. pickettii* CCUG3314 (DDDE), *P. aeruginosa* ATCC 27853 (EEFH), and *S. maltophilia* RIVM 96-330 (CCCD). Lane MW, molecular weight (MW) standard.

testing was particularly disappointing in the past (34). Difficulties and inaccuracies at the level of laboratory procedures have been documented even when simple plating procedures are used for screening (15). Mix-ups with relatively nonpathogenic bacterial species have been described (21), and even commercially available diagnostic tests have failed (6). Also, the taxonomy of *Burkholderia* spp. is still evolving, and the pathogenic potential of the diverse species and subspecies for humans has not been elucidated yet (10). Major biological and genetic diversity among isolates of *B. cepacia* subspecies cultured from the same environmental source can be demonstrated (7). Variation in the flagellin genes of *B. cepacia* can be demonstrated for subdivisions within the species as well (14). In addition, it has been demonstrated that clinical isolates of *B. cepacia* may have characteristics of *B. gladioli* (3), a species for which some records of severe infections exist as well (1, 13, 17, 20, 28, 30). The issues mentioned here warrant in-depth studies on the value of the currently available diagnostic tools for the identification of *B. cepacia*.

In the present communication, we tried to define the best procedures for diagnosing the presence of *Burkholderia* spp. in the sputum of CF patients. A collection of *Burkholderia* strains was obtained from several reference laboratories. Based on our results, we suggest the use of either BCSA or OFPBL plates for the initial isolation of *B. cepacia* directly from clinical material. The sensitivity of these growth media appeared to be excellent (96 and 100%, respectively); the specificity, however, was not 100%. Because of the growth of species other than *B. cepacia* on selective agar, such agar cannot be used for the definitive identification of a strain as *B. cepacia* but can provide a useful first screen, as stated previously (15). In the presence of colonization or infection by *B. gladioli*, many strains will not be detected if this procedure is used as the single diagnostic assay.

It was quite striking to find that the automated assays, such

FIG. 2. Prediction of different restriction sites in the ribosomal genes for *Burkholderia* spp. Numbering identifies nucleotides in the consensus small-subunit rRNA gene sequences available through GenBank. Restriction sites are highlighted by boxes, and the nature of the restriction enzyme is indicated by the appropriate abbreviation.

as Vitek and MicroScan, performed with varying but consistently insufficient accuracy. Only the Vitek GNI reliably identified most of the *B. cepacia* strains, but it encountered major problems with *B. gladioli* strains. Improvement in both cards and software is certainly needed for all automated systems currently available. The major outcome of the present analysis is the fact that molecular identification by PCR-RFLP analysis is superior to the biochemical and microbiological species identification procedures used here, although it should be emphasized that the API 20NE performed satisfactorily, as documented previously (15, 27). No international gold standard for the routine laboratory identification of clinically relevant *Burkholderia* species exists to date, so the definition of accurate sensitivities and specificities for the tests used in the present communication remains a topic for future investigations. The PCR-RFLP procedure was found to be in excellent agreement with strain identification provided by the different study participants.

Genetic mosaicism has been proposed on the basis of phenotypic studies for *B. cepacia* and *B. gladioli* (3). On the basis of the currently known small-subunit rRNA gene sequences (Fig. 2) and the PCR-RFLP test, intraspecific polymorphism can be anticipated and noted, respectively. Depending on the restriction enzyme, species-specific RFLP patterns or RFLP patterns that are shared between the two species can be observed. Whether this observation relates to genetic exchange or mere coincidence, based on lack of variability in the ribosomal region that is targeted by the enzyme, requires additional DNA sequencing studies. In the present study, no clearly overlapping characteristics were noted among the diverse types of the strains and the tests that were performed. Only for a *B. cepacia* strain with type ABBB was the PCR with primer pair PC1-PC2 positive, although exceptions may be encountered in the future. It would be interesting to determine whether the RFLPs coincide with the epidemicity and pathogenicity of the strain (24, 25).

For adequate characterization of the strains of *Burkholderia* spp. described in this report, we suggest that the PCR-RFLP procedure be used as the most definitive means of identification. However, it must be emphasized that PCR-based methods are not generally available to the clinical microbiologist. We recommend that microbiologists initiate characterization with a diagnostic agar followed by API 20NE. Routine microbiology procedures should be used prior to the tests mentioned above in order to exclude all nonrelevant bacterial species.

The PCR-RFLP procedure seems to be a reliable tool for discriminating strains of *B. cepacia* versus *B. gladioli*. This technique should facilitate more detailed studies on the clinical relevance of the latter species in CF and other diseases. Whereas previously a comparison of the endotoxicity of lipopolysaccharide isolated from both species was a measure of clinical relevance (10), now precise species identification can replace superficial lipopolysaccharide characteristics for a more species-defining feature. In order to make another important step forward, the DNA assays used here and those newly described in the literature (2) should be adapted for PCR directly from sputum samples.

REFERENCES

1. **Barker, P. M., R. E. Wood, and P. H. Gilligan.** 1997. Lung infection with *Burkholderia gladioli* in a child with cystic fibrosis: acute clinical and spirometric deterioration. Pediatr. Pulmonol. **23:**123–125.
2. **Bauernfeind, A., I. Schneider, R. Jungwirth, and C. Roller.** 1998. Discrimi-

nation of *Burkholderia* species detectable in cystic fibrosis patients by PCR. J. Clin. Microbiol. **36:**2748–2751.

3. **Baxter, I. A., P. A. Lambert, and I. N. Simpson.** 1997. Isolation from clinical sources of *Burkholderia cepacia* possessing characteristics of *Burkholderia gladioli*. J. Antimicrob. Chemother. **39:**169–175.

4. **Bevivino, A., S. Tabacchioni, L. Chiarini, M. V. Carusi, M. del Gallo, and P. Visca.** 1994. Phenotypic comparison between rhizosphere and clinical isolates of *Burkholderia cepacia*. Microbiology **140:**1069–1077.

5. **Boom, R., C. J. A. Sol, M. M. M. Salimans, C. L. Jansen, P. M. E. Wertheim-van Dillen, and J. van der Noordaa.** 1990. Rapid and simple method for purification of nucleic acids. J. Clin. Microbiol. **28:**495–503.

6. **Burdge, D. R., M. A. Noble, M. E. Campbell, V. L. Krell, and D. P. Speert.** 1995. *Xanthomonas maltophilia* misidentified as *Pseudomonas cepacia* in cultures of sputum from patients with cystic fibrosis: a diagnostic pitfall with major clinical implications. Clin. Infect. Dis. **20:**445–448.

7. **Di Cello, F., A. Bevivino, L. Chiarini, R. Fani, D. Paffetti, S. Tabacchioni, and C. Dalmastri.** 1997. Biodiversity of a *Burkholderia cepacia* population isolated from the maize rhizosphere at different plant growth stages. Appl. Environ. Microbiol. **63:**4485–4493.

8. **Gilligan, P. H.** 1991. Microbiology of airway disease in patients with cystic fibrosis. Clin. Microbiol. Rev. **4:**35–51.

9. **Gillis, M., T. van Van, R. Bardin, M. Goor, P. Hebbar, A. Willems, P. Segers, K. Kersters, T. Heulin, and M. P. Fernandez.** 1995. Polyphasic taxonomy in the genus *Burkholderia* leading to an amended description of the genus and proposition of *Burkholderia vietnamiensis* sp. nov. for N₂-fixing isolates from rice in Vietnam. Int. J. Syst. Bacteriol. **45:**274–289.

10. **Govan, J. R. W., and V. Deretic.** 1996. Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. Microbiol. Rev. **60:**539–574.

11. **Govan, J. R. W., J. E. Hughes, and P. Vandamme.** 1996. *Burkholderia cepacia*: medical, taxonomic and ecological issues. J. Med. Microbiol. **45:**395–407.

12. **Govan, J. R. W., P. H. Brown, J. Maddison, C. J. Doherty, J. W. Nelson, M. Dodd, A. P. Greening, and A. K. Webb.** 1993. Evidence for transmission of *Pseudomonas cepacia* by social contact in cystic fibrosis. Lancet **342:**15–19.

13. **Graves, M., T. Robin, A. M. Chipman, J. Wong, S. Khashe, and J. M. Janda.** 1997. Four additional cases of *Burkholderia gladioli* infection with microbiological correlates and review. Clin. Infect. Dis. **25:**838–842.

14. **Hales, B. A., J. A. W. Morgan, C. A. Hart, and C. Winstanley.** 1998. Variation in flagellin genes and proteins of *Burkholderia cepacia*. J. Bacteriol. **180:**1110–1118.

15. **Henry, D. A., M. E. Campbell, J. J. LiPuma, and D. P. Speert.** 1997. Identification of *Burkholderia cepacia* isolates from patients with cystic fibrosis and use of a simple new selective medium. J. Clin. Microbiol. **35:**614–619.

16. **Henry, D. A.** Personal communication.

17. **Hoare, S., and A. J. Cant.** 1996. Chronic granulomatous disease presenting as severe sepsis due to *Burkholderia gladioli*. Clin. Infect. Dis. **23:**411.

18. **Holmes, A., J. Govan, and R. Goldstein.** 1998. Agriculture use of *Burkholderia (Pseudomonas) cepacia*: a threat to human health? Emerg. Infect. Dis. **4:**221–227.

19. **Kanji, S. S., V. Tapson, R. D. Davis, J. Madden, and I. Browning.** 1997. Infections in patients with cystic fibrosis following lung transplantation. Chest **112:**924–930.

20. **Khan, S. U., S. M. Gordon, P. C. Stillwell, T. J. Kirby, and A. C. Arroliga.** 1996. Empyema and bloodstream infection caused by *Burkholderia gladioli* in a patient with cystic fibrosis after lung transplantation. Pediatr. Infect. Dis. J. **15:**637–639.

21. **Kiska, D. L., A. Kerr, M. C. Jones, J. A. Carracciolo, B. Eskridge, M. Jordan, S. Miller, D. Hughes, N. King, and P. H. Gilligan.** 1996. Accuracy of four commercial systems for identification of *Burkholderia cepacia* and other gram-negative nonfermenting bacilli recovered from patients with cystic fibrosis. J. Clin. Microbiol. **34:**886–891.

22. **Landa A. S., F. M. Sipkema, J. Weijma, A. A. Beenackers, J. Dolfing, and D. B. Janssen.** 1994. Cometabolic degradation of trichloroethylene by *Pseudomonas cepacia* G4 in a chemostat with toluene as the primary sub-strate. Appl. Environ. Microbiol. **60:**3368–3374.

23. **LiPuma, J. J.** 1998. *Burkholderia cepacia*: management issues and new insights. Clin. Chest Med. **19:**473–486.

24. **Mahenthiralingam, E., D. A. Simpson, and D. P. Speert.** 1997. Identification and characterization of a novel DNA marker associated with epidemic *Burkholderia cepacia* strains recovered from patients with cystic fibrosis. J. Clin. Microbiol. **35:**808–816.

25. **Mahenthiralingam, E., M. E. Campbell, D. A. Henry, and D. P. Speert.** 1996. Epidemiology of *Burkholderia cepacia* infection in patients with cystic fibrosis: analysis by randomly amplified polymorphic DNA fingerprinting. J. Clin. Microbiol. **34:**2914–2920.

26. **O'Callaghan, E. M., M. S. Tanner, and G. J. Boulnois.** 1994. Development of a PCR probe test for identifying *Pseudomonas aeruginosa* and *Pseudomonas (Burkholderia) cepacia*. J. Clin. Pathol. **47:**222–226.

27. **Pitt, T. L., and J. R. W. Govan.** 1993. *Pseudomonas cepacia* and cystic fibrosis. PHLS Microbiol. Dig. **10:**69–72.

28. **Ross, J., S. M. Holland, V. J. Gill, E. S. DeCarlo, and J. I. Gallin.** 1995. Severe *Burkholderia (Pseudomonas) gladioli* infection in chronic granulomatous disease: report of two successfully treated cases. Clin. Infect. Dis. **21:**1291–1293.

29. **Segonds, C., T. Heulin, N. Marty, and G. Chabanon.** 1999 Differentiation of *Burkholderia* species by PCR-restriction fragment analysis of the 16S rRNA gene and application to cystic fibrosis isolates. J. Clin. Microbiol. **37:**2201–2208.

30. **Shin, J. H., S. H. Kim, M. G. Shin, S. P. Suh, D. W. Ryang, and M. H. Jeong.** 1997. Bacteremia due to *Burkholderia gladioli*: case report. Clin. Infect. Dis. **25:**1264–1265.

31. **Smith, D. L., L. B. Gumery, E. G. Smith, D. E. Stableforth, M. E. Kaufmann, and T. L. Pitt.** 1993. Epidemic of *Pseudomonas cepacia* in an adult cystic fibrosis unit: evidence of person-to-person transmission. J. Clin. Microbiol. **31:**3017–3022.

32. **Sun, L., R. Jiang, S. Steinbach, A. Holmes, C. Campanelli, J. Forstner, U. Sajjan, Y. Tan, M. Riley, and R. Goldstein.** 1995. The emergence of a highly transmissible lineage of abl+ *Pseudomonas (Burkholderia) cepacia* causing CF centre epidemics in North America and Britain. Nat. Med. **1:**661–665.

33. **Tabacchioni, S., P. Visca, L. Chiarini, A. Bevinino, C. di Serio, S. Fancelli, and R. Fani.** 1995. Molecular characterisation of rhizosphere and clinical isolates of *Burkholderia cepacia*. Res. Microbiol. **146:**531–542.

34. **Tablan, O. C., L. A. Carson, L. B. Cusick, L. A. Bland, W. J. Martone, and W. R. Jarvis.** 1987. Laboratory proficiency test results on use of selective media for isolating *Pseudomonas cepacia* from simulated sputum specimens of patients with cystic fibrosis. J. Clin. Microbiol. **25:**485–487.

35. **Tyler, S. D., C. A. Strathdee, K. R. Rozee, and W. M. Johnson.** 1995. Primers designed to differentiate pathogenic pseudomonads on the basis of the sequencing of genes coding for 16S-23S rRNA internal transcribed spacers. Clin. Diagn. Lab. Immunol. **4:**448–453.

36. **Vandamme, P., G. Holmes, M. Vancanneyt, T. Coenye, B. Hoste, R. Coopman, H. Revets, S. Lauers, M. Gillis, K. Kersters, and J. R. V. Govan.** 1997. Occurrence of multiple genomovars of *Burkholderia cepacia* in cystic fibrosis patients and proposal of *Burkholderia multivorans* sp. nov. Int. J. Syst. Bacteriol. **47:**1188–1200.

37. **Weisburg, W. G., S. M. Barns, D. A. Pelletier, and D. J. Lane.** 1991. 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol. **173:**697–703.

38. **Welch, D. F., M. J. Muszynski, C. H. Pai, M. J. Marcon, M. M. Hribar, P. H. Gilligan, J. M. Matsen, P. A. Ahlin, B. C. Hilman, and S. A. Chartrand.** 1987. Selective and differential medium for recovery of *Pseudomonas cepacia* from the respiratory tracts of patients with cystic fibrosis. J. Clin. Microbiol. **25:**1730–1734.

39. **Yabuuchi, E., Y. Kosako, I. Yano, H. Hotta, and Y. Nishiuchi.** 1995. Transfer of two *Burkholderia* and an *Alcaligenes* species to *Ralstonia* gen. nov.: proposal of *Ralstonia pickettii* (Ralston, Palleromi and Doudoroff 1973) comb. nov., *Ralstonia solanacearum* (Smith 1896) comb. nov. and *Ralstonia eutropha* (Davis 1969) comb. nov. Microbiol. Immunol. **39:**897–904.