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Burkholderia cepacia Complex Infection in Italian Patients with Cystic Fibrosis: Prevalence, Epidemiology, and Genomovar Status

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The prevalence, epidemiology, and genomovar status of *Burkholderia cepacia* complex strains recovered from Italian cystic fibrosis (CF) patients were investigated using genetic typing and species identification methods. Four CF treatment centers were examined: two in Sicily, one in central Italy, and one in northern Italy. *B. cepacia* complex bacteria were isolated from 59 out of 683 CF patients attending these centers (8.6%). For the two geographically related treatment centers in Sicily, there was a high incidence of infection caused by a single epidemic clone possessing the *cblA* gene and belonging to *B. cepacia* genomovar III, *recA* group III-A, closely related to the major North America-United Kingdom clone, ET12; instability of the *cblA* sequence was also demonstrated for clonal isolates. In summary, of all the strains of *B. cepacia* encountered in the Italian CF population, the genomovar III, *recA* group III-A strains were the most prevalent and transmissible. However, patient-to-patient spread was also observed with several other genomovars, including strains of novel taxonomic status within the *B. cepacia* complex. A combination of genetic identification and molecular typing analysis is recommended to fully define specific risks posed by the genomovar status of strains within the *B. cepacia* complex.

Bacteria of the *Burkholderia cepacia* complex have been increasingly isolated as pathogens from cystic fibrosis (CF) patient populations due to their capacity for spread between patients, and their potential role in declining lung function with necrotizing pneumonia and frequently fatal septicemia, the so-called *B. cepacia* syndrome, has also been noted. Published reports indicate that different *Burkholderia* strains, identified as *B. cepacia* by conventional laboratory procedures, may be associated with a poor clinical prognosis for some individuals and/or with enhanced person-to-person transmissibility (13). Cross-infection between CF patients and epidemic outbreaks have been documented both within and outside hospitals (8, 10). Various markers have been associated with transmissible strains of *B. cepacia*, such as extracellular appendages known as cable pili (7, 20, 23) and a conserved 1.4-kb open reading frame called *esmR* (*B. cepacia* epidemic strain marker [16]).

The taxonomic diversity and the peculiar genomic characteristics of these organisms present diagnostic laboratories with many problems (18, 28). The name "*B. cepacia* complex" was proposed to comprise a cluster of five closely related species, originally referred to as *B. cepacia* genomovars I through V (6, 29). Recent research has also defined genomovar VI as a new member of the *B. cepacia* complex which shares considerable similarity with *Burkholderia multivorans* (4), and the name *Burkholderia ambifaria*, for bacteria belonging to genomovar VII (5), has been proposed. Determination of the genomovar status of *B. cepacia* complex strains is based on a polyphasic taxonomic approach encompassing traditional phenotypic and genotypic tests (27, 28, 29). To simplify the identification process, recent genetic procedures based on nucleotide sequence polymorphisms of the 16S rRNA gene (1, 13, 14, 21) or the *recA* gene (18) have been developed. Rapid and precise identification of bacteria is essential to evaluate specific risks, in terms of clinical prognosis and epidemicity, posed by each genomovar within the *B. cepacia* complex. Our study was performed in order to (i) evaluate the prevalence of *B. cepacia* complex infection in CF patients attending four Italian treatment centers over a period of 10 months, (ii) identify the genomovar status of each isolate, (iii) study the epidemiological and genetic relatedness of *Burkholderia* isolates, and (iv) evaluate the frequency of transmissibility markers and their association with the epidemiological classification of the strains.

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MATERIALS AND METHODS

Bacterial strains and culture. From September 1998 to July 1999, 683 patients were screened for *B. cepacia* complex infection, by sputum cultures on oxidation-fermentation base polymyxin B agar (Becton Dickinson). Strains were presumptively identified by the API 20NE (Bio Merieux) system; positive cultures were then sent to our reference laboratory for further characterization. A total of 92 *B. cepacia* isolates were obtained from the respiratory tract of patients attending the following CF centers: 28 were from 9 CF patients from Catania, Sicily; 33 were from 33 CF patients hospitalized in Palermo, Sicily; 1 isolate was from 1 CF patient in Gualdo Tadino, central Italy; and 30 were from 22 CF patients in Milan, northern Italy.

Six control strains, all isolated from CF patients in Vancouver, British Columbia, Canada, were obtained from a previously published collection (11, 16, 19, 23). Additional control strains for each current genomovar and *recA* gene restriction fragment length polymorphism (RFLP) type were also included (17, 18).

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TABLE 1. 16S rRNA gene-based identification and characterization of *Burkholderia* isolates from the four Italian centers

Center	No. of strains					
	16S rRNA-PCR identification				$cblA^+$	$esmR^+$
	Total no.	*Burkholderia* genomovars I, III, and IV	*B. multivorans*/ *B. vietnamiensis*	*B. gladioli*		
Catania	28	27	0	1	9	6
Palermo	33	33	0	0	24	15
Gualdo Tadino	1	1	0	0	0	1
Milan	30	28	1	1	0	18
Total	92	89	1	2	33	40

One reference isolate (ATCC 25608) was obtained from the American Type Culture Collection.

Genomovar status identification based on the rRNA genes. Preliminary identification of genomovar status was performed on the basis of a previously published PCR-based procedure for the identification of sequence motifs within the 16S and 23S ribosomal DNAs (1). Three separate PCRs were run, including primers of different degrees of specificity, in order to achieve stepwise exclusion of single species: Ce-16-2_{1028} excluded *B. multivorans, Burkholderia vietnamiensis,* and *Burkholderia gladioli*; Mu-Vi-16-2_{1028} excluded *B. cepacia*; and Gl-16-2_{457} excluded *B. cepacia, B. multivorans,* and *B. vietnamiensis.*

Genomovar status identification based on the *recA* gene. A total of 53 strains, representative of the genetic diversity within the collection (determined by pulsed-field gel electrophoresis [PFGE]; see below), were examined (18). Briefly, identification of *B. cepacia* complex was carried out using PCR with primers which amplify the entire *recA* gene of bacteria within the *B. cepacia* complex. Genomovar status was then identified by RFLP of the amplified *recA* gene and confirmed using PCR primers specific for each genomovar. Strains that produced novel RFLP types and that tested negative with the genomovar-specific primers were subjected to nucleotide sequence analysis of the upstream region of the *recA* gene using PCR primers. Phylogenetic analysis of the resulting sequences was then used to place these strains within the complex. In addition, RFLP analysis of the 16S rRNA operon was performed on these strains to confirm their subclassification within genomovar I, III, or IV, *B. multivorans,* or *B. vietnamiensis*.

PFGE. DNA fingerprinting by PFGE was carried out by the method of Grothues et al. (9). In brief, isolates were grown overnight on nutrient agar and then suspended in 1 ml of SE buffer (75 mM NaCl, 25 mM EDTA, pH 7.5) and adjusted to 10^{10} CFU/ml. Cell suspensions were mixed with an equal volume of 1.6% low-melting-point agarose, molded into plugs at 4°C, and lysed at 56°C overnight; the DNA inserts were then digested with *Spe*I, according to the supplier's instructions (New England Biolabs). Macrorestriction fragments were separated using a Gene Navigator apparatus (Pharmacia Biotech) at 10°C for 19 h, with a start time of 5 s and an end-pulse time of 35 s, at a field strength of 6 V/cm. A concatemer ladder of lambda phage DNA was used as a size marker. Interpretation of genomic relatedness was performed using well-established criteria (22, 26).

PCR detection of *cblA* and *esmR*. Whole-cell DNA was amplified in a reaction mixture consisting of 200 μM deoxynucleoside triphosphates, 1 μM (each) primer, 1× PCR buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.3], 1.5 mM $MgCl_2$), 2.5 mM $MgCl_2$, and 1 U of *Taq* DNA polymerase. Each mixture was overlaid with 50 μl of liquid paraffin and placed in a PT 100 thermal cycler. The primer sequences used to amplify the *esmR* gene were as previously described (16). DNA sequences were amplified as follows: 30 cycles of 1 min at 94°C, 1 min at 62°C, and 1 min at 72°C and a final extension step at 72°C for 5 min. For the amplification of the *cblA* gene, primers were as previously described (20). Amplification was done for 30 cycles, each consisting of 1 min at 94°C, 1 min at 58°C, and 1 min at 72°C.

RESULTS

Prevalence of *B. cepacia* complex bacteria. Our study involved 683 out of 2,717 patients (25%) included in the National Italian CF Register up to the end of 1995 (2, 25), under microbiological surveillance in CF treatment centers. From the initial biochemical screening performed in the four centers, the prevalence of *B. cepacia* infection was 9.5% overall (65 out of 683 patients): 30% (9 out of 30 patients) in Catania, 16.5% (33 out of 200 patients) in Palermo, 4% (1 out of 23 patients) in Gualdo Tadino, and 5% (22 out of 430 patients) in Milan. Further molecular characterization based on the 16S rRNA and *recA* gene (see below) indicated that isolates from six of these patients were not of the *B. cepacia* complex, providing a final prevalence of infection of 8.6% (59 out of 683 patients). Misidentification of *B. cepacia* complex was associated with all four CF treatment centers participating in this survey (Catania, Palermo, and Gualdo Tadino each contributing one misidentified strain and Milan contributing three non-*B. cepacia* complex bacteria).

Identification of *Burkholderia* spp. based on the 16S rRNA gene. A total of 92 isolates were identified as *B. cepacia* after routine biochemical tests. Preliminary molecular identification based on the 16S rRNA gene was performed on all 92 CF isolates. Briefly, 89 of these 92 isolates (97%) were found to belong to *B. cepacia* genomovar I, III, or IV, these genomovars not being discriminated. One strain (1%) was identified as *B. multivorans* or *B. vietnamiensis,* and two (2%) were *B. gladioli* (Table 1).

Identification of *B. cepacia* complex genomovars based on the *recA* gene. Due to the inability of the 16S rRNA gene PCR to distinguish all the current genomovars, the nucleotide sequence polymorphism in the *recA* gene was analyzed to determine specific genomovar status (Table 2). Examination of the PCR-RFLP patterns of the *recA* gene was performed first. Ten distinct *Hae*III-derived RFLP patterns were found among 53 isolates that were representative of the genetic diversity of the *B. cepacia* complex strains present at the four CF centers (see

TABLE 2. Comparison between 16S rRNA- and *recA*-based identification of *Burkholderia* isolates

Genomovar or species	No. of strains								
	16S rRNA-based identification	*recA*-based identification							
		Genomovar or species							Not *B. cepacia* complex
		I	*B. multivorans*	III-A	III-B	*B. stabilis*	*B. vietnamiensis*	I or III	
Burkholderia genomovars I, III, and IV	89	3	4	50	13	3	0	12	4
B. multivorans/*B. vietnamiensis*	1	0	0	1	0	0	0	0	0
B. gladioli	2	0	0	0	0	0	0	0	2
Total	92	3	4	51	13	3	0	12	6

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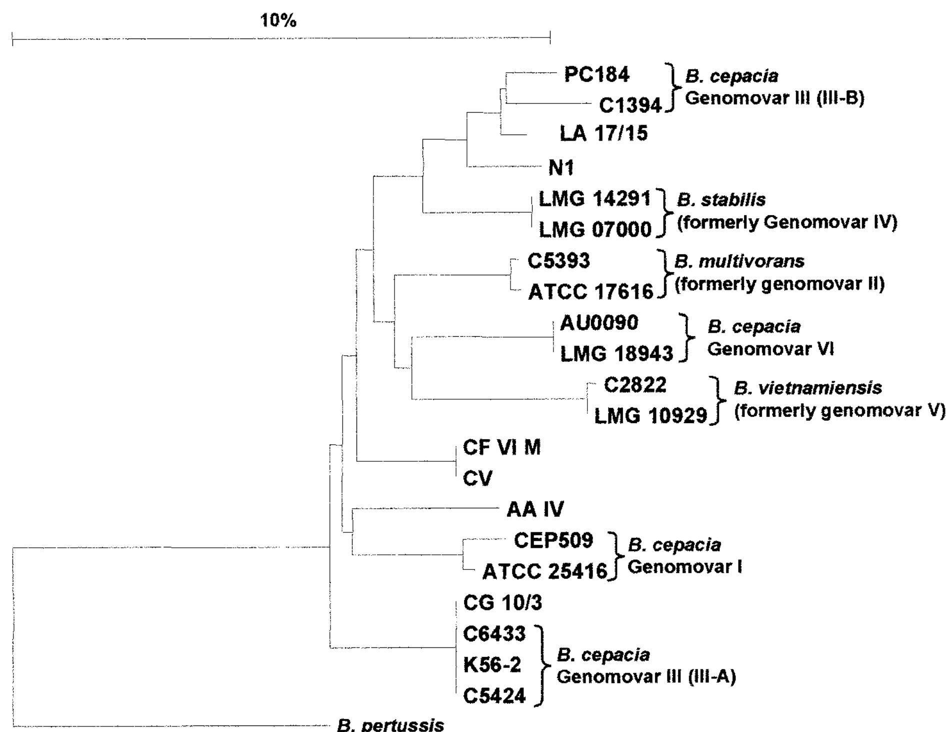

FIG. 1. Phylogenetic tree of the *B. cepacia* complex based on the *recA* gene. The location of the Catania cable pilus-encoding strain within genomovar III-A is shown (represented by strain CG 10/3). Multiple sequence alignment was performed, and the tree was rooted with the published *recA* sequence from *Bordetella pertussis*. Genetic distance is indicated by the scale.

PFGE results below). Six of these RFLP patterns correlated with those described previously, and their genomovar status was then confirmed using the genomovar-specific primers and was as follows: RFLP type E, genomovar I; RFLP type F, *B. multivorans*; RFLP type G, genomovar III (*recA* group III-A); RFLP types H and I, genomovar III (*recA* group III-B); and RFLP J, *Burkholderia stabilis*. Four *recA* gene RFLP types were not previously described and were designated RFLP types AZ, I3, S, and U. Apart from amplification of the entire *recA* gene with the *B. cepacia* complex-specific primers BCR1 and BCR2, strains possessing these novel RFLP types failed to react with any of the genomovar-specific *recA* PCR primers. To confirm the status of these strains as members of the *B. cepacia* complex, nucleotide sequence analysis of the 5′ 527-bp region of the *recA* gene was performed and phylogenetically analyzed. All strains possessing novel *recA* RFLP types were members of the *B. cepacia* complex based on *recA* phylogenetic analysis. The resulting phylogenetic tree is shown in Fig. 1. However, using current methods these strains have been found to have indeterminate genomovar status and may be novel taxonomic groups within the *B. cepacia* complex. To confirm this *recA*-based result, RFLP analysis of the 16S rRNA gene was also performed. All strains of *recA* RFLP types AZ, I3, S, and U possessed the 16S rRNA gene RFLP pattern 2 (data not shown). This 16S rRNA gene RFLP is shared by the control strains of genomovar I, genomovar III, *B. ambifaria* (genomovar VII), and *B. stabilis*. However, none of these novel strains possessed other phenotypic or genetic characteristics associated with *B. ambifaria* or *B. stabilis*; hence, they were identified as *B. cepacia* complex genomovar I-III indeterminate status (Fig. 1).

Final prevalence of each *B. cepacia* complex genomovar. Final genomovar status identification was attributed based on the *recA* polymorphism; the results, in comparison with the 16S rRNA gene analysis, are summarized in Table 2. *B. cepacia* genomovar III was the dominant species present among the Italian isolates examined (69.5%, 64 out of 92 isolates), and of these, the majority (80%, 51 out of 64 isolates) belonged to *B. cepacia* genomovar *recA* group III-A.

Genome macrorestriction analysis. On the basis of PFGE-based RFLP analysis, the epidemiology of *B. cepacia* strains was assessed. Serial isolates recovered from the same patients produced conserved macrorestriction patterns, demonstrating chronic persistence of individual strain types in 10 patients and recurrence of infection in 2 patients (data not shown).

Macrorestriction analysis enabled the identification of at least 27 different clones responsible for the spread of *B. cepacia* infection. Different epidemiological features, showing cross-transmission or sporadicity of infection, were present in each center (Table 3). A predominant clone was identified in the two Sicilian centers. This "Sicilian epidemic clone" (PFGE strain type A) chronically infected 26 of the 42 *B. cepacia* complex-positive patients (62%) attending the two treatment centers. This highly transmissible strain was not found outside Sicily. Its PFGE profile was closely related (similarity coefficient, 78%) to the C5424 reference strain, a member of the ET12 North America-United Kingdom transatlantic clone (Fig. 2A).

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TABLE 3. Numbers of patients colonized with cross-transmitted or sporadic strains of different genomovars or species and PFGE types

Center	No. of patients colonized with strain of genomovar or species and PFGE type																												
	Cross-transmitted strains									Sporadic strains																			
	I XB	III-A		III-B			*B. stabilis*, S	I-III		*B. multivorans*			III-A									III-B		*B. stabilis*, I	I-III				
		A[b]	P	T	XE	UA		Q	BA	SB	L	ND[a]	B[b]	C[b]	H	SA	X	Y	Z	U	ND*	G	V		D[c]	E[c]	F[c]	R	UB
Catania		3									1		1	1	1						1	1		1	1	1	1		
Palermo		23				2			2			1								1			1						1
Milan	3		2	2	2		2	2		1						1	1	1	1									1	

[a] ND, not determined. DNA was shared in repeated samples.
[b] Three strains, one each of types A, B, and C, were isolates from the same patient.
[c] Three strains, one each of types D, E, and F, were isolates from the same patient.

PCR detection of *cblA* and *esmR*. Within this study, the presence of published molecular markers of *B. cepacia* complex transmissibility was demonstrated for the first time within the Italian CF population (Table 1). Cable pilus-associated sequences were present in a total of 33 of the 92 isolates, all from the Sicilian centers, belonging to the *B. cepacia* genomovars III and I-III. Notably, the epidemic outbreak involving the two Sicilian centers was sustained by 26 different variants of the same genomovar III-A clone: 14 were *cblA* positive and *esmR* negative, 7 were *cblA* positive and *esmR* positive, 3 were *cblA* negative and *esmR* positive, and 2 were *cblA* negative and *esmR* negative (Fig. 2B). A total of 43 out of the 59 patients were colonized by *B. cepacia* complex strains as a result of epidemic spread or cross-transmission: for 23 of these patients, the strains involved were shown to bear the *cblA* gene, while the remaining 20 strains were *cblA* negative.

The presence of *esmR* sequences was shown for 40 of the 92 isolates examined, and all of these belonged to *B. cepacia* genomovar III: 22 of these *esmR*-positive strains were associated with epidemic spread, while the remaining 18 were not. Instability of both transmissibility markers within a single genomovar III-A clone was also observed (Fig. 2).

Correlation between bacterial genomovar status and the epidemicity of infection. Using the genomovar status of each strain obtained by analysis of the *recA* gene, a correlation between the risk of patient-to-patient cross-infection and genomovar status was made. The highest correlation between cross-infection and genomovar was associated with strains of the *B. cepacia* complex genomovar III, *recA* group III-A. There were 43 patients sharing the same strain as a result of cross-infection: in 28 of these cases, the strain involved belonged to genomovar III, *recA* group III-A. A relative risk of cross-infection with genomovar III-A was calculated as 1.9 (28 cases associated with genomovar III-A compared to 15 cases associated with strains of the other genomovars). *recA* group III-A included epidemic CF strains from the cable pilus-encoding lineage (18, 20) and the Vancouver outbreaks (15, 17). Phylogenetic analysis of the *recA* gene sequence confirmed that the Catania cable pilus-carrying strain, responsible for the Sicilian epidemic, was within the same genetic cluster and closely related to these epidemic genomovar III-A strains (Fig. 1). A second genomovar III-A strain, of distinct PFGE type, was also responsible for cross-infection of two patients. Other instances of patients sharing the same strains were observed for *B. cepacia* of all the other genomovars except for *B. multivorans* (Table 3).

DISCUSSION

The worldwide increase of *B. cepacia* infection in CF patients suggests its epidemic spread, but the source and transmissibility of strains involved remain controversial. It has been suggested elsewhere that strains of the *B. cepacia* complex are not equally transmissible; rather, there exist highly transmissible lineages, presumably of a clonal nature, and heterogeneous lineages of negligible transmissibility (23). We investigated the hypothesis that transmissibility might be associated with a particular taxonomic group, genomovar or species, within the *B. cepacia* complex.

From our screening, the prevalence of infection of *B. cepacia* complex in CF patients in Italy was 8.6% overall, 18.3% in Sicily and 5% in Milan. The prevalence of *B. cepacia* infection, as reported in a survey published in 1997, was 3.8%, clearly underestimated, since at that time only a few laboratories used the selective culture media for isolation (24). A higher prevalence (20.5%) was reported from the Campania region, southern Italy, in 1999, when appropriate microbiological proce-

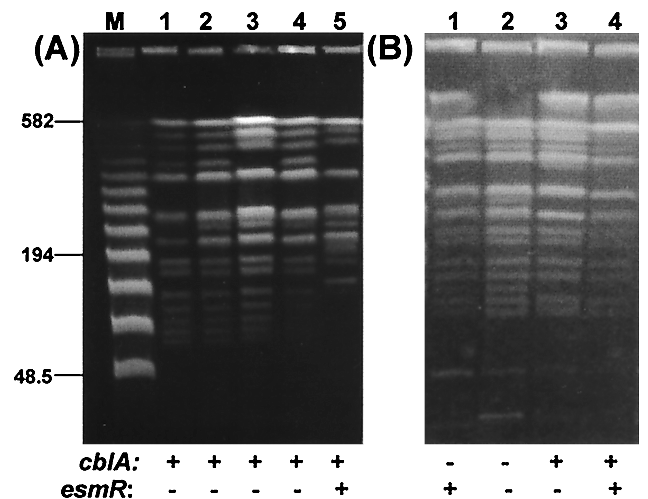

FIG. 2. PFGE-macrorestriction profiles of different *cblA* or *esmR* variants of the Sicilian epidemic clone. (A) Lanes 1 to 4, isolates from different Sicilian patients; lane 5, ET12 strain C5424 (16). (B) Lanes 1 to 4, *cblA* or *esmR* variants of the epidemic clone from different Sicilian patients. A λ ladder molecular size marker was run in the lane labeled M, and the size of relevant bands is indicated in kilobases. The presence (+) or absence (−) of the *cblA* and *esmR* markers is indicated below each lane.

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dures were used, although identification was based on standard laboratory procedures (30).

Analysis of the *recA* gene of the *B. cepacia* complex is a more discriminatory molecular approach than the analysis of the 16S rRNA gene to identify isolates and evaluate the specific risk posed by infection with a given genomovar and the epidemic spread of infection. We evaluated this risk as being about twice that for infection sustained by microorganisms of genomovar III, *recA* group III-A, with respect to other strains of the complex. This group included the Sicilian epidemic clone, which was phylogenetically related to the ET12 North America-United Kingdom transatlantic clone.

Although infection of the majority of patients involved in the Sicilian outbreak was sustained by strains possessing the *cblA* gene, a genetic marker associated with transmissibility, 5 of the 26 patients involved were actually colonized with a *cblA*-negative variant of the same clone, suggesting that the region may be subject to some instability. The PFGE fingerprints of these *cblA*-negative variants of the type clone showed minor changes in their banding profile, which may be associated with genome rearrangement and loss of the *cblA* gene. Early studies of the *B. cepacia* genome showed pronounced genome plasticity due to its multiple replicon organization and large numbers of insertion sequences (12, 29). In light of the heavy use of genetic markers for the epidemiological management of *B. cepacia* complex infections in CF (3, 16, 20, 23), the stability of these markers must be better understood.

Our findings suggest that the *esmR* and the *cblA* sequences may be encoded on a chromosomal region which is unstable in some *B. cepacia* genomovar III epidemic strains. Genetic instability has been well documented for the *esmR* open reading frame (16, 18), while instability of the *cblA* gene had not been previously demonstrated.

In conclusion, we have demonstrated that genomovar III is the most prevalent genomovar of *B. cepacia* complex bacteria among a population of Italian CF patients as previously reported (28). We also observed CF infection and patient-to-patient spread of *B. cepacia* complex bacteria of novel taxonomic status. Genetic identification of bacteria recovered from clinical settings as well as from other sources is essential to further understanding of the transmissibility and pathogenic potential of different genomovars or species within the *B. cepacia* complex.

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