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

ANTONELLA AGODI, ESHWAR MAHENTHIRALINGAM, MARTINA BARCHITTA, VIVIANA GIANNINO, AGATA SCIACCA, and STEFANIA STEFANI

Department of Biomedical Sciences, Department of Microbiological and Gynecological Sciences, and Microbiological Laboratory, Policlinico, University of Catania, Catania, Italy, and School of Biosciences, Cardiff University, Cardiff, Wales, United Kingdom

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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 csmR (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.

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 Guado Tedino, 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).

* Corresponding author. Mailing address: Section of Microbiology of the Department of Microbiological and Gynecological Sciences, University of Catania, Via Androne 81, 95124 Catania, Italy. Phone: 39 (095) 311352. Fax: 39 (095) 325032. E-mail: stefanis@mbox.unict.it.
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 RNAs (1). Three separate PCRs were run, including primers of different degrees of specificity, in order to achieve stepwise exclusion of single species: Ce-16-2028 excluded *B. multivorans*, *Burkholderia vietnamiensis*, and *Burkholderia gladioli*; Mu-Vi-16-2028 excluded *B. cepacia*; and Gl-16-2028 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 Gro-...
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 sporadity 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 CS424 reference strain, a member of the ET12 North America-United Kingdom transatlantic clone (Fig. 2A).
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 epidemiology 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).

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

<table>
<thead>
<tr>
<th>Center</th>
<th>Cross-transmitted strains</th>
<th>Sporadic strains</th>
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<tr>
<td></td>
<td>I-XB</td>
<td>II-B</td>
</tr>
<tr>
<td></td>
<td>I-XB</td>
<td>II-B</td>
</tr>
<tr>
<td>Catania</td>
<td>3</td>
<td>23</td>
</tr>
<tr>
<td>Palermo</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Milan</td>
<td>3</td>
<td>2</td>
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*ND, not determined. DNA was shared in repeated samples.
*Three strains, one each of types A, B, and C, were isolates from the same patient.
*Three strains, one each of types D, E, and F, were isolates from the same patient.

**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-
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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