Revised Approach for Identification of Isolates within the
Burkholderia cepacia Complex and Description of
Clinical Isolates Not Assigned to Any of
the Known Genomovars

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One hundred thirty-eight clinical isolates of the Burkholderia cepacia complex (Bcc) were identified using a modified strategy that involved PCR detection of the cblA gene for the ET12 lineage simultaneously with detection of the Bcc recA PCR product; recA sequence cluster analysis also was part of the strategy. Four strains could not be assigned to any of the known genomovars.

Organisms of the Burkholderia cepacia complex (Bcc) are important opportunistic pathogens in persons with cystic fibrosis (CF) that are capable of causing life-threatening respiratory tract infections (10). The complex consists of at least nine recognized species or genomovars, namely, B. cepacia (genomovar I), B. multivorans (genomovar II), B. cenocepacia (genomovar III), B. stabilis (genomovar IV), B. vietnamiensis (genomovar V), B. dolosa (genomovar VI), B. ambifaria (genomovar VII), B. anthina (genomovar VIII), and B. pyroccinia (genomovar IX). Genomovar III (B. cenocepacia) is further divided into at least four phylogenetic lineages (IIIA, IIIB, IIC, and IID) (18). Reliable identification is important, since some genomovars, particularly the ET12 lineage of genomovar IIIA (15), are associated with high transmissibility between patients and a poor prognosis (7). B. multivorans and B. cenocepacia account for the majority of isolates from CF patients, with patient-to-patient spread mainly being associated with genomovars IIIA (8, 12) and IIIB (3, 6), although reports also describe epidemic spread of lineages of genomovar IID (13) and B. dolosa (1, 2). The highest mortality rate has been associated with genomovar IIIA, but reports have now appeared that suggest that genomovar IID and B. dolosa are similarly virulent (2, 9, 13).

Identification usually is performed by DNA-based methods, exploiting sequence differences in the recA gene to assign isolates to the appropriate genomovar. This is achieved by PCR using primers specific for the Bcc, restriction fragment length polymorphism (RFLP) analysis of the product, and further PCR with a series of genomovar-specific primers, as described by Mahenthiralingam et al. (11) and other authors (5, 19). However, with the substantial number of genomovars and subgenomovars now described, it has become cumbersome to use PCR to try to detect them all. RFLP results sometimes are ambiguous, and patterns not previously described sometimes are obtained (19). Occasionally, isolates that cannot be assigned to any of the genomovars by recA sequencing are received, but these may be misidentified on the basis of a faint cross-reaction in one or more of the genomovar-specific PCRs or from the RFLP result. The ET12 lineage, associated with epidemic spread and, in some cases, high mortality, may be missed, because often it does not produce a strong band in the initial Bcc PCR.

To address these problems, we have devised a modified scheme that involves initial detection of the Bcc multiplexed with detection of cblA, the cable pilus gene (an excellent marker for the ET12 lineage), followed by screening for B. multivorans using the genomovar II species-specific PCR. The identities of cblA-positive isolates are confirmed by carrying out the PCR for genomovar IIIA, and those of genomovar II-positive isolates are confirmed by RFLP; the presence of a band of the expected size (378 bp) or an RFLP pattern compatible with B. multivorans, respectively, confirms the identifications. The Bcc ampicillins of the remaining isolates are subjected to sequencing and are identified both by BLAST searches (http://www.ncbi.nlm.nih.gov/BLAST/) and by clustering on a dendrogram, which compares the sequences to that of an 897-bp fragment. recA sequences of isolates known to belong to each genomovar, described in publications and available from GenBank, are included in the database, and isolates simply cluster with the appropriate genomovar. As sequences of more isolates are added, a better estimate of the extent of sequence diversity within each genomovar is obtained, so that cutoffs can be used to make an informed decision as to whether an isolate clusters closely enough with any of the known genomovars. In this way, we have identified some clinical isolates that cannot be assigned. We provide an identification service for CF centers in the United Kingdom and have received multiple submissions of one such strain from a patient over a period of 3 years, indicating that these isolates can be associated with chronic infection.

Clinical and reference isolates were subjected to multiplex PCR for the Bcc and cblA using the BCR1/BCR2 primers (11) and primers for cblA (mpcableF148, 5′-CTGAATACGTTCCGATCGC-3′; and mpcableR419, 5′-GGGCTCGTCTG

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TCTTCA-3'), designed from consensus sequences from alignments of GenBank accession numbers AF455151 to AF455162, AY114293, AY082893, AJ304454, and U10244. The cblA amplicon is 272 bp in size. Previously described cblA primers (4) were not suitable for this multiplex, since their melting temperatures differ considerably from those of BCR1 and BCR2. PCRs were carried out in 25-μl volumes containing 1× PCR buffer (containing 1.5 mM MgCl₂) (QIAGEN, Crawley, United Kingdom), an additional 1.5 mM MgCl₂ (for a final total concentration of 3 mM), 1× Q solution (QIAGEN), 250 μM of each deoxynucleoside triphosphate, 1.5 U Taq DNA polymerase, and 10 pmol of each primer. Conditions were 35 cycles of 30 s at 94°C, 45 s at 63°C, and 1 min at 72°C, followed by a final extension at 72°C for 10 min. Genomovars II and IIIA were detected by PCR as described above using the BCRBM1/BCRBM2 and BCRG3A1/BCRG3A2 primer pairs, respectively (11). RFLP and pulsed-field gel electrophoresis (PFGE) were carried out as described previously (11, 16). BCR1/BCR2 amplicons were purified using QIAquick PCR purification columns (QIAGEN) according to the manufacturer’s instructions and were sequenced using the BCR1 (forward reaction) and BCR2 (reverse reaction) primers with the Beckman Coulter dye terminator cycle-sequencing quick-start kit (High Wycombe, United Kingdom). Reaction products were separated and analyzed on a Beckman Coulter CEQ8000 sequencer. Sequences of an 897-bp fragment, corresponding to nucleotides 75 to 971 of GenBank accession number AF143788, were compared by pairwise alignment with a BioNumerics database (Applied Maths, Kortrijk, Belgium), and dendrograms were prepared that show the percentage of similarity using the unweighted-pair group method using average linkages for clustering.

Inclusion of the multiplex cblA primers in the Bcc PCR was found to be effective in identifying all representatives of the ET12 lineage tested. That they were representatives of ET12 was confirmed both by a positive result in the genomovar IIIA-specific PCR and by their characteristic PFGE banding pattern. These isolates gave, at best, only a faint amplicon for the Bcc, which could have been missed if detection of cblA had not been included at this initial stage. It gave the same results as those obtained using the PCR for the cblA gene described by Clode et al. (4), which previously had been used in our laboratory. The multiplex cblA primers also can be used in combination with the BCRG3A1/BCRG3A2 primers (used for detection of genomovar IIIA).

Following the initial PCR, subsequent screening of all Bcc-positive or cblA-positive isolates using the species-specific PCRs for genomovars II and IIIA identified most of our clinical isolates. The BCR1/BCR2 recA amplicons of the remainder were sequenced. Most of these were found to belong to genomovar I, IIIB, or V (Fig. 1). Some isolates of B. multivorans, the ET12 lineage of B. cenocepacia, and a strain of IIIA that was distinct from ET12 and negative for the cblA gene (IIIAcblAneg) also were sequenced, as were reference isolates LMG 19230 (genomovar IIIC), LMG 21462 (genomovar IIID), LMG 10929 (B. vietnamiensis), LMG 18941 (B. dolosa), LMG 20983 (B. anthina), and LMG 14191 (B. pyrocinia). This procedure provided information for assessing sequence diversity within genomovars and tested the method to see if particular known genomovars could be detected. A cutoff

![FIG. 1. Dendrogram comparing sequences of an 897-bp recA fragment of isolates belonging to the Bcc. Dotted lines indicate 98.5 and 97.6% similarity. Isolate designations are those given in the GenBank entry for the accession number cited. Example isolates from this study are labeled according to their identification, e.g., BCG1 1 is B. cepacia, BM1 to -3 are B. multivorans, and BC3B 1 to -3 are B. cenocepacia genomovar IIIB, and reference isolates are labeled with their LMG numbers. isolates BCCU1 to BCCU4 represent strains that could not be assigned to any of the known genomovars.](http://jcm.asm.org/Downloaded/from/fig1c.png)

![FIG. 2. PFGE profiles of XbaI-digested genomic DNA of isolates of genomovar IIIB (BC3B 1 to BC3B 8) received from the eight patients with this lineage during the study period. BC3B 6 and BC3B 7 were from siblings. All of the remaining isolates were from patients from different centers.](http://jcm.asm.org/Downloaded/from/fig2c.png)
level of 98.5% was adopted, above which we could confidently assign an isolate to a genomovar. For genomovars VIII and IX, however, a lower cutoff was more appropriate, since the sequences of different isolates of these genomovars in GenBank clustered only within 97.6% and 98.2% similarity, respectively (Fig. 1). All of the reference isolates we tested were identified correctly using the appropriate cutoff. Four clinical isolates (BCCU1 to BCCU4) did not cluster closely enough by our criteria with any of the known genomovars, and three of them (BCCU1, BCCU2, and BCCU4) could not be assigned, even with a cutoff of 97.6%. We have received 12 isolates from the CF patient from whom BCCU1 was isolated, all of which have identical PFGE profiles, over a period of 3 years. The remaining isolates were recent (2006 or 2007), single submissions from separate patients. BCCU3 and BCCU4 were both from non-CF patients; the patient with BCCU3 had traveled to Sri Lanka. All of these isolates also were identified as Burkholderia spp. by other methods (gas chromatography of fatty acids and partial 16S rRNA gene sequencing).

Over the 6 months from October 2006 to March 2007, during which this method was developed and evaluated, the laboratory received 80 isolates of genomovar II (from 64 patients), 31 isolates of genomovar IIIA (24 of which [from 16 patients] were PCR positive for the cblA gene), 10 isolates of genomovar IIIB (from 8 patients), 14 isolates of genomovar V (from 9 patients), and 3 isolates of genomovar I (from 2 patients) from CF centers in the United Kingdom. Eight isolates could not be assigned; these were representatives of BCCU1 to BCCU4. Comparison, by PFGE, of the isolates of genomovar V and of the cblA-negative isolates of genomovar IIIB showed that each patient harbored his or her own strain, while all of the cblA-positive isolates were representatives of ET12. The isolates of genomovar IIIB consisted of six PFGE types, with two pairs of patients sharing the same strain as one another and one pair being from siblings (Fig. 2). Representatives of genomovars II and IIIA remain the most common among patients with CF in the United Kingdom, with many belonging to the ET12 lineage of genomovar IIIA. To date, we have not detected isolates of genomovars IID and VI, which have been associated with high virulence and transmissibility in other countries. The use of this revised procedure should enable us to provide effective surveillance and to rapidly identify any emerging genomovars affecting CF patients from whom we receive isolates. It should prevent misidentifications, and other problems, which have been reported by researchers using only a PCR/RFLP approach (14, 17, 19). Some clinical isolates that are PCR positive for the Bcc cannot be assigned to a genomovar but may be clinically significant.

**Nucleotide sequence accession numbers.** Partial recA sequences of isolates BCCU1 to BCCU4 were deposited in GenBank under accession numbers EF408665, EF408666, EF408667, and EF522189, respectively.

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**REFERENCES**


