SNaPBcen: a Novel and Practical Tool for Genotyping Burkholderia cenocepacia

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Burkholderia cenocepacia is the most prevalent and feared member of the Burkholderia cepacia complex in lung infections of cystic fibrosis (CF). Genotyping and monitoring of long-term colonization are critical at clinical units; however, the differentiation of specific lineages performed by multilocus sequence typing (MLST) is still limited to a small number of isolates due to the high cost and time-consuming procedure. The aim of this study was to optimize a protocol (the SNaPBcen assay) for extensive bacterial population studies. The strategy used for the SNaPBcen assay is based on targeting single nucleotide polymorphisms (SNPs) located in MLST genes instead of sequencing full MLST sequences. Nonpolymorphic and redundant MLST positions were eliminated, and a set of 24 polymorphisms included in the SNaPBcen assay ensures a high-resolution genomic characterization. These polymorphisms were identified based on the comparative analysis of 137 B. cenocepacia MLST profiles available online (http://pubmlst.org/bcc/). The group of 81 clinical isolates of B. cenocepacia examined in this study using the SNaPBcen assay revealed 51 distinct profiles, and a final discriminatory power of 0.9997 compared with MLST was determined. The SNaPBcen assay was able to reveal isolates with microvariations and the presence of multiple clonal variants in patients chronically colonized with B. cenocepacia. Main phylogenetic subgroups IIIA, IIIB, and IIIC of B. cenocepacia could be separated by the G994R polymorphism included in the panel. The SNaPBcen assay proved to be a rapid and robust alternative to the standard MLST for B. cenocepacia, allowing the simultaneous analysis of multiple polymorphisms following amplification and mini-sequencing reactions.
PCR that amplifies target genomic regions containing the polymorphisms, which are subsequently detected by mini-sequencing. The mini-sequencing is performed using single base extension primers and fluorescently labeled deoxy nucleoside triphosphates (ddNTPs). By employing primers with different lengths (nucleotide tails can be added at 5′), this practice allows the recognition of fragments of multiple sizes by automated capillary electrophoresis. The results appear as a plot with different colored peaks that represent site-specific genomic variations. A previous study already proposed a SNP multiplex assay for identification of some Bcc species by targeting six polymorphisms in the gyrB gene (16). However, the differentiation of specific lineages is still performed by MLST. A SNP-based strategy has recently been developed for genotyping of Pseudomonas aeruginosa, presenting a discriminatory power of 0.9993 in comparison with MLST (19).

Thus, aiming to optimize a method able to overcome the limitations of MLST to identify lineages and genotype large number of bacterial isolates, in the present work we developed a SNP-based method for genotyping B. cenocepacia, here designated the SNaPBcen assay. B. cenocepacia is the most clinically relevant species within the complex; therefore, it was the focus of the present study. The standard MLST method for Bcc required the sequencing analysis of 2,377 nucleotides, but only some of these positions present relevant genotyping information. Nonpolymorphic positions, as well as redundant polymorphic positions, were eliminated. Finally, the method targeted a set of 24 polymorphisms located in six MLST genes and the assortment of the most informative nucleotide positions allowed a high-resolution genomic characterization of bacterial isolates. Thus, the proposed method represents a practical approach for genotyping the isolates of B. cenocepacia, allowing extensive microbial population studies and monitoring of long-term colonization.

MATERIALS AND METHODS

B. cenocepacia isolates and DNA extraction. Eighty-one isolates of B. cenocepacia recA lineages IIIA and IIIB, obtained at both the Portuguese CF Treatment Center at Santa Maria Hospital (HSM) in Lisbon and Hospital S. João in Oporto, were used in this study (see Table S1 in the supplemental material). The HSM isolates were obtained during the past 18 years, as part of the hospital routine, from respiratory secretions of CF patients and were characterized molecularly and phenotypically at the IBB/IST laboratory (20–24). A small group of non-cenocepacia species of the Bcc, B. cepacia (n = 10), B. contaminans (n = 5), B. dolosa (n = 6), B. multivorans (n = 5), and B. stabilis (n = 4), was also included in the present study. B. cenocepacia isolates and other bacterial isolates were kept frozen at −80°C and cultured on Pseudomonas isolation agar (Difco) medium plates before DNA extraction. Single colonies of each isolate were suspended in 4 ml of Luria-Bertani broth (Difco) medium and grown overnight with orbital agitation (250 rpm) at 37°C. Total genomic DNA was extracted from B. cenocepacia isolates, using a cell and tissue kit (Gentra Systems; Qiagen). The concentrations of genomic DNA solutions and quality parameters (260/280 nm and 260/230 nm) were estimated using an ND-1000 Spectrophotometer (NanoDrop). Finally, two DNA purification steps were performed with ethanol (70 [vol/vol]) were added to the protocol. Bacterial DNA was finally suspended in 50 μl of ultrapure water and stored at −20°C.

MLST genotyping and sequencing analysis. Seven fragments of housekeeping genes (atpD, gdhB, gyrB, recA, lepA, phaC, and trpB) used for MLST were amplified in B. cenocepacia and non-cenocepacia isolates (10). The suggested primers were employed, and new primers were designed for cases with amplification problems employing Primer3 (v0.4.0; http://frodo.wi.mit.edu/) (25). Subsequently, hairpin and primer-dimer secondary structures were avoided by using AutoDimer v1 (http://www.cstl.nist.gov/biotech/strbase/AutoDimer Homepage/AutoDimerProgramHomepage.htm) (26). The final set of primers employed for amplification of MLST gene fragments is shown in Table S2 in the supplemental material. Touchdown PCR was conducted using a final volume of 5.0 μl, containing 2.5 μl of multiplex PCR master mix (Qiagen), 0.5 μl of bacterial DNA (50 to 250 ng), 0.5 μl of primer mix (each one at 2 μM), 0.5 μl of Q-solution (Qiagen), and 1.0 μl of ultrapure water. PCR conditions were as follows: denaturation for 15 min at 95°C; 26 cycles with denaturation for 1 min at 95°C, primer annealing for 30 s at 57°C (recA), 60°C (atpD, gdhB, lepA, phaC, and trpB), or 64°C (gyrB), and extension for 2 min at 72°C; and final extension for 10 min at 72°C. Before the primer annealing, touchdown PCR with three successive cycles (total of nine cycles) at increasing temperatures was performed. Finally, amplicon sizes were confirmed after separation by polyacrylamide gel electrophoresis and standard silver-staining detection (27).

PCR fragments were purified with ExoSap-IT (Escherichia coli exonuclease I and shrimp alkaline phosphatase; USB Corporation), according to the manufacturer’s instructions. Sequencing reactions were carried out using an ABI BigDye Terminator cycle sequencing ready reaction kit (Applied Biosystems). The sequencing reactions included an incubation for 2 min at 96°C followed by 35 cycles with denaturation for 15 s at 96°C, primer annealing for 9 s at 50°C, extension for 2 min at 60°C, and then 10 min at 60°C. The product was purified using Sephadex (Exapnsys) columns and a volume of 8.0 μl HiDi formamide (Applied Biosystems) added to the product before sequencing analysis on an ABI Prism 3100 genetic analyzer (Applied Biosystems).

SNaPBcen assay. The strategy used for the SNaPBcen assay is based on targeting SNPs located in MLST genes instead of sequencing full MLST sequences. A nonhomologous tail was added at the 5′ end of extension primers to allow the separation by capillary electrophoresis. The final size of each primer plus the nonhomologous tail ranged from 17 to 116 bp (Table 1). In silico prevention of hairpin and primer-dimer secondary structures was performed by using AutoDimer v1. The SNaPBcen assay was carried out in a final volume of 5 μl containing 1.5 μl of PCR product (purified with ExoSap-IT as described above), 1 μl of SNaPBcen primer mix (each one at 1 μM), 1 μl of ABI Prism SNaPshot multiplex kit (Applied Biosystems), and 1.5 μl of ultrapure water. The reaction mixture was run with 25 extension cycles under the following conditions: denaturation for 10 s at 96°C, primer annealing for 5 s at 50°C, and extension for 30 s at 60°C. Unincorporated ddNTPs were removed with 1 U of SAP (shrimp alkaline phosphatase; USB Corporation) following incubation for 1 h at 37°C and 15 min at 85°C as suggested by the manufacturer. SNaPBcen products (0.5 μl) were mixed with 9.0 μl of HiDi formamide and 0.5 μl of GeneScan-120 LIZ size standard (Applied Biosystems). Electrophoresis was performed on a 3130xl Genetic Analyzer (Applied Biosystems) using the filter set E5, and the data were analyzed with the software GeneMapper v4.0 (Applied Biosystems).

Data and statistical analysis. The MLST profiles of 137 online entries of B. cenocepacia (downloaded from http://pubmlst.org/bcc/) were deposited in an in-house record that included the MLST data of bacteria sequenced in this study. Network analysis was performed with the Network 4.6.1.0 program (www.fluxus-engineering.com/sharenet.htm) (28). Procedures to determine minimum spanning trees were performed at the MLST website (http://pubmlst.org/rel/rel/mlstanalyse/mlstanalyse.pl/site =pubmlst&kpage=mskRefe Ree=pubmlst.org). Statistical analysis was performed using Arlequin 3.1 software (http://cmpg.unibe.ch/software/arlequin3/) (29) and Microsoft Office Excel 2010 (Microsoft Corporation). Simpson’s diversity index was applied to determine the discriminatory power of individual SNPs and of the proposed multiplex strategy.

RESULTS

Amplification of MLST gene fragments. The primers described by Baldwin et al. (10) and Spilker et al. (30) to be used in the...
TABLE 1 Primers used for SNaPBcen assay

<table>
<thead>
<tr>
<th>Name</th>
<th>Expected SNP</th>
<th>Primer sequence (5’ to 3’)</th>
<th>Final length (bp)</th>
<th>Expected heterozygosity (%)</th>
<th>Garza-Williamson index</th>
</tr>
</thead>
<tbody>
<tr>
<td>At125F</td>
<td>C/T</td>
<td>GCGGATCATGAGACGTGCT</td>
<td>108 (109–111)</td>
<td>0.08028</td>
<td>0.66667</td>
</tr>
<tr>
<td>At1358R</td>
<td>A/C/G/T</td>
<td>ATCGAAGCGTCACGTGCT</td>
<td>72 (73–74)</td>
<td>0.50682</td>
<td>1.00000</td>
</tr>
<tr>
<td>At248F</td>
<td>C/T</td>
<td>TCGAAGCGGTATCAGGT</td>
<td>116 (117–118)</td>
<td>0.03092</td>
<td>0.66667</td>
</tr>
<tr>
<td>At255R</td>
<td>C/T</td>
<td>TGCGAAGCGGCAGTGCT</td>
<td>112 (113–114)</td>
<td>0.13591</td>
<td>0.66667</td>
</tr>
<tr>
<td>At365R</td>
<td>C/G/T</td>
<td>ACCGAGAATGAGACGTGCA</td>
<td>75 (76–77)</td>
<td>0.23020</td>
<td>0.75000</td>
</tr>
<tr>
<td>At443F</td>
<td>C/G</td>
<td>TGCGATGCGCTTGGCGGCT</td>
<td>64 (65–67)</td>
<td>0.02072</td>
<td>1.00000</td>
</tr>
<tr>
<td>Gy43R</td>
<td>C/T</td>
<td>ACTTTCCGGGCGTGGTAACAC</td>
<td>82 (84–85)</td>
<td>0.51631</td>
<td>1.00000</td>
</tr>
<tr>
<td>Gy53R</td>
<td>A/C/G</td>
<td>CTATTCAAGCAGACAGCC</td>
<td>61 (62–63)</td>
<td>0.09975</td>
<td>1.00000</td>
</tr>
<tr>
<td>Gy101F</td>
<td>A/C/G/T</td>
<td>CCGGAGCGGAAAGACGCG</td>
<td>95 (97–99)</td>
<td>0.37418</td>
<td>1.00000</td>
</tr>
<tr>
<td>Gy149R</td>
<td>C/G/T</td>
<td>GACACTTCAAGCCGTCACAY</td>
<td>90 (92–94)</td>
<td>0.33895</td>
<td>1.00000</td>
</tr>
<tr>
<td>Gy218F</td>
<td>C/T</td>
<td>CGCGCCACCGGAAGTGCA</td>
<td>28 (31–33)</td>
<td>0.49820</td>
<td>0.66667</td>
</tr>
<tr>
<td>Gy230R</td>
<td>C/G/T</td>
<td>ATCGAAGCGGCAGGGCA</td>
<td>95 (97–99)</td>
<td>0.37418</td>
<td>1.00000</td>
</tr>
<tr>
<td>Gy251R</td>
<td>C/T</td>
<td>CGCGGAGATGCGATTGACAC</td>
<td>29 (36–38)</td>
<td>0.01042</td>
<td>0.66667</td>
</tr>
<tr>
<td>Gy323F</td>
<td>C/G/T</td>
<td>AAGCCGTTGGCGGATCGC</td>
<td>44 (47–49)</td>
<td>0.50649</td>
<td>1.00000</td>
</tr>
<tr>
<td>Gy338R</td>
<td>A/C/T</td>
<td>AAATCTTCTTGTGCCTGCA</td>
<td>98 (100–102)</td>
<td>0.44290</td>
<td>0.75000</td>
</tr>
<tr>
<td>Gy374R</td>
<td>C/G/T</td>
<td>ATGTACGAGCGAAGCCCTCAC</td>
<td>86 (88–90)</td>
<td>0.60106</td>
<td>1.00000</td>
</tr>
<tr>
<td>Gy428R</td>
<td>A/C/G</td>
<td>CGCGATGCGGCAGAAGATGCGA</td>
<td>57 (59–61)</td>
<td>0.50671</td>
<td>1.00000</td>
</tr>
<tr>
<td>Le390R</td>
<td>C/G</td>
<td>GATGACGATCGGTTCGCA</td>
<td>36 (42–44)</td>
<td>0.39218</td>
<td>1.00000</td>
</tr>
<tr>
<td>Ph500R</td>
<td>A/G</td>
<td>GTGAAGGCTCGATCGGCA</td>
<td>49 (54–57)</td>
<td>0.01042</td>
<td>0.66667</td>
</tr>
<tr>
<td>Ph536F</td>
<td>A/C/G/T</td>
<td>ATCGAAGCGGCACCGTGACAC</td>
<td>103 (104–105)</td>
<td>0.51684</td>
<td>1.00000</td>
</tr>
<tr>
<td>Ph536R</td>
<td>C/T</td>
<td>TGGCGAGCGCAGGCTAGGAC</td>
<td>80 (81–82)</td>
<td>0.05099</td>
<td>0.66667</td>
</tr>
<tr>
<td>Re21R</td>
<td>A/C/G</td>
<td>TTGCACGACACCGATCGC</td>
<td>66 (69–71)</td>
<td>0.52252</td>
<td>1.00000</td>
</tr>
<tr>
<td>Re195F</td>
<td>A/C/G/T</td>
<td>GTGCTCCGAGATCGGTGACAC</td>
<td>105 (105–107)</td>
<td>0.47835</td>
<td>1.00000</td>
</tr>
</tbody>
</table>

*Expected SNP data represent the predictable SNPs on MLST profiles (5’ to 3’).*
**SNaPBcen assay for genotyping of *B. cenocepacia* isolates.**

The SNaPBcen assay ensured an electrophoretic profile with 24 peaks for *B. cenocepacia* isolates distinguished by automated capillary electrophoresis (Fig. 2). The SNaP profile could then be obtained to facilitate comparison with MLST data, as shown in Fig. 2. The assay was further tested in a group of 81 clinical isolates of *B. cenocepacia* (see Table S1 in the supplemental material), revealing 51 distinct SNaP profiles. Further sequencing of MLST genes in the group of isolates with similar SNaP profiles showed the absence of polymorphisms in 98% of the isolates—a single isolate could not be differentiated by the SNaPBcen assay. All clinical isolates with similar SNaP profiles corresponded to isolates collected from individual patients during several years of chronic infections; sequential isolates obtained from the same patient also showed the same recA RFLP profile and ribotype pattern (20, 22, 23) and had been considered clonal variants, presumably resulting from adaptive evolution in the CF lung during chronic infection (20). No isolates with similar SNaP profiles were obtained from two distinct patients. Therefore, the proposed methodology was reproducible and able to distinguish the MLST profiles in our collection of *B. cenocepacia*. Figure 3 shows the distribution of the SNaP profiles observed in this study versus the SNaP profiles of the isolates collected worldwide whose MLST profiles are described in the MLST database. The collection of isolates experimentally tested in this study was not found grouped in the network far from the isolates previously reported worldwide; in fact, the genotypes were found dispersed along the network. A few isolates could be grouped according to genotyping similarity (G1 to G5 in Fig. 3) and corresponded to isolates with microevolutions or a few genetic differences in tested markers, most of the time obtained from the same patient. These cases were observed in previously characterized recA lineages IIIA and IIIB. The SNaPBcen assay was able to distinguish sequential clonal variants presenting a few genotyping differences retrieved from eight patients with long-term *B. cenocepacia* colonization (patients AB, AN, G, J, O, P, R, and T described in Table S1 in the supplemental material).

Occasionally, the patients were shown to be colonized simultaneously with two genetically very distinct strains, as in the case of patient O (Fig. 4). Thirteen isolates obtained from patient O between 1998 and 2002 were included in this study and resulted in nine distinct SNaP profiles (four were characterized as IIIA and five as IIIB and are marked O_IIIA and O_IIIB, respectively, in Fig. 4). Isolates AB_A1, AB_A2, O_A1, and R_B1 showed additional mutations moving these isolates far from the groups AB, O_IIIA, and R; nevertheless, these isolates do have the same subgroup, recA RFLP profile, and ribopattern as all the patient group isolates.

**Population analysis of *B. cenocepacia* isolates and their recA lineages.** The main phylogenetic subgroups of recA lineages IIIA, IIIB, and IIIC of *B. cenocepacia* could be separated by the polymor-
phism Gl94R included in our panel, revealing a T, C, and G, respectively. The rare subgroup IIID was similar to IIIB in the marker Gl94R; however, it could be distinguished by combining markers At158R and Gy374R (IIID isolates presented A and G, respectively, while these positions never showed this profile in IIIB isolates). In silico analysis of the sequence types described at the MLST database agreed with this description. Therefore, the SNaPBcen assay could easily separate *B. cenocepacia* isolates of this study into the subgroups IIIA (n = 41) and IIIB (n = 40).

We analyzed a set of 137 unique online ST entries of *B. cenocepacia* isolates (obtained from pubMLST in November 2012) obtained from different countries between 1961 and 2012 plus the group of 51 SNaP profiles emerging from this study. SNaP profiles of isolates from Australia, the United States, Canada, and New Zealand were compared with the profiles in the complete group by network analysis and application of the minimum spanning tree method; similar subpopulations were selected for the temporal intervals 1961 to 1999 and 2000 to 2012. No subdivisions of isolates could be observed in either the spatial or temporal data sets, while these positions never showed this profile in IIIB isolates). In the column listing isolation dates, "00" represents the year 2000, "01" represents the year 2001, etc.

**FIG 3** (A) Network distribution of SNaP profiles obtained from this study (black circles) among the profiles in the complete list obtained from the MLST database. G1 to G5 represent five groups of closely related SNaP profiles observed in our collection of isolates. (B) List of isolates and clonal variants considered in each of the closely related groups and their characteristics. In the column listing isolation dates, "00" represents the year 2000, "01" represents the year 2001, etc.

**DISCUSSION**

The experimental strategy adopted in this study introduces an innovative methodology for genotyping *B. cenocepacia* based on the analysis of 24 SNPs located at the MLST genes. The SNaPBcen assay represents a practical and robust alternative to the standard MLST test used for *B. cenocepacia*, especially for large collections of isolates, allowing the simultaneous analysis of multiple polymorphisms following amplification and mini-sequencing reactions. This method uses neutral genetic polymorphisms which are important for understanding the diversity and dispersal mechanisms of this bacterium worldwide and could be used to improve the diagnosis and surveillance of *B. cenocepacia* in hospital laboratories. The newly proposed method can cost six to seven times less than MLST and produces genotyping results in few hours.

*SNaPBcen* is a fast and reproducible assay and represents a valuable method for daily routine in clinical laboratories. A small number of alternatives are presently available for the genome analysis of Bcc species, especially of *B. cenocepacia*, most of them
being not useful and reflecting a need for high-throughput molecular approaches for epidemiological analysis. In 2011, the analysis of 35 profiles of MLST suggested the reduction of the number of housekeeping genes from seven to six (by removal of lepA) in B. cenocepacia analyses to promote the cost-effectiveness of this strategy (31). The assay proposed in the present study uses less than 0.008% of the total number of nucleotide sequences used in MLST in a single mini-sequencing reaction. The small number of polymorphisms tested with SNaPbcen considerably reduces the genotyping data analysis burden and the number of errors observed with some ambiguous MLST sequences. In addition, the present assay is able to differentiate the recA lineages IIIA, IIIB, IIIC, and IID of B. cenocepacia. Remarkably, for this species only, among a group of isolates of different Bcc isolate species, the complete SNaP profiles with 24 markers can be observed. The development of proper SNP genotyping panels for other Bcc species would also be advantageous for rapid analysis. Genotyping of B. cenocepacia may lastly become a low-cost test with all the subsequent benefits being valuable in cases of patients colonized or infected by multiple strains, as was the case of patient O reported in this study.

The analysis of our collection of isolates resulted in a set of SNaP profiles that showed no spatial or temporal subpopulations identified following network analyses, therefore corroborating the assumption of epidemic B. cenocepacia bacteria previously described as spreading within CF populations in Canada and Europe (4). Several patients were found to be chronically colonized with the same B. cenocepacia clone, and the sequential isolates produced similar or closely related SNaP profiles. In fact, microvariation was frequently described in clinical Bcc isolates recovered sequentially from a CF patient during long-term infection, as previously reported (20, 21, 32). These cases support the idea of the previously described plasticity of the B. cenocepacia genome with respect to accumulation of alterations and adaptation to a stressful pulmonary environment (33). During chronic colonization, bacteria experience changing pressures exerted by the host environment, in particular, changes in nutrient availability, challenges of the immune defenses and antimicrobial therapy, and oxygen limitation (34–36). These changes may lead to the emergence of phenotypic variants of the underlying clonal population (20, 24, 37–39) and decreased production of virulence factors associated with acute infections aiding immune evasion (35, 39, 40). We have

FIG 4 (A) Network distribution of SNaP profiles observed in patients AB (black circles), O (dark gray circles), and R (light gray circles) among the profiles in the complete list characterized by SNaPbcen in this work. AB_A1, AB_A2, O_A1, and R_B1 represent Burkholderia cenocepacia isolates; O_IIIA and O_IIIB represent the groups of isolates of recA IIIA and IIIB found in patient O. (B) List of isolates considered in patients and their characteristics; isolates with similar SNaP profiles are represented in a single circle.
noted in the present report that the emergence of *B. cenocepacia* clonal variant isolates showed several alterations in the SNaP profile during long-term infection and the progression of the disease. Rapid bacterial adaptation may be the consequence of antibiotic therapy or the presence of free radicals and/or the result of immune selective pressures (34–36). This is not a new assumption, as an increasing mutation rate has been described in other bacteria under similar stressful conditions (39, 41–43), but it supports the idea that this assay is suitable for differentiation of clonal variants with identical ribopatterns.

Thus, the SNaPBcen assay is a powerful tool for definition of subgroups and genotyping *B. cenocepacia* strains, particularly for genotyping large collections of isolates. Furthermore, the flexibility of the method allows the modification and addition of extra markers in case they prove to be relevant for specific populations. This advantage may become very important, as progress in the generation of sequence data (44) can substantially increase the number of target polymorphisms useful for *B. cenocepacia* genotyping. An online SNP platform associated with the former and successful MLST database may complement and facilitate the analysis of the genotyping data available for this species. The SNaPBcen assay is suitable for rapid genetic analysis of large collections of *B. cenocepacia* isolates and employs limited resources and funds in comparison with the standard methods.

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We declare that we have no conflicts of interest.

**REFERENCES**


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