Direct PCR Detection of *Burkholderia cepacia* Complex and Identification of Its Genomovars by Using Sputum as Source of DNA

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Received 15 April 2002/Returned for modification 16 May 2002/Accepted 23 June 2002

We developed a nested PCR assay that detects the *recA* gene of the *Burkholderia cepacia* complex in sputum. The product of the first PCR round is also used to identify the genomovar of the pathogen. The protocol achieves high sensitivity and specificity with simple interpretation of genomovar status.

Burkholderia cepacia is a multiresistant and transmissible opportunistic pathogen that frequently impairs the clinical state of patients with cystic fibrosis (CF) (1, 3, 9, 10). The group of *B. cepacia* complex (BCC) organisms consists of eight genomovars associated with different levels of virulence and patient-to-patient transmissibility (2, 15): *B. cepacia* genomovar I, *B. multivorans* (genomovar II), *B. cepacia* genomovar III, *B. stabilis* (genomovar IV), *B. vietnamiensis* (genomovar V), *B. cepacia* genomovar VI, *B. ambifaria* (genomovar VII), and *B. pyrocinia* (5). To reduce the probability of BCC spreading among patients with CF, a reliable early test that detects small quantities of the bacteria in clinical samples is needed. Conventional microbiological diagnostics of the BCC based on the results of culture and subsequent biochemical identification is insufficient for this purpose because of the potential risk of misidentification or false negativity (7). Moreover, these methods are not able to reliably distinguish the genomovars of the BCC (21).

To increase the sensitivity and specificity of diagnostic routines and to identify the genomovars, use of molecular genetic methods is inevitable. Two target genes are commonly used for BCC analysis: the 16S rRNA gene (11, 19, 20) and the *recA* gene (13, 16). The *recA* gene polymorphisms enable both differentiation of the BCC from other closely related bacteria and its sorting into genomovars. Moreover, the differences in the *recA* sequences within genomovar III led to the establishment of two *recA* clusters designated III-A and III-B.

The present study sought (i) to develop a rapid diagnostic method for early detection of BCC organisms and determination of their genomovars directly from sputum and (ii) to assess the occurrence of the BCC genomovars in Czech patients with CF.

From May 2001 to April 2002, we collected 211 sputum samples from 134 consecutive CF patients attending the Prague CF center (55 males, 79 females; age range, 0 to 33 years). All samples were examined for the presence of the BCC by culture and our novel PCR protocol. For cultivation, speci-

mens were liquefied with a homogenization solution (0.9% NaCl, 50 mM KH₂PO₄, 35 mM NaOH, 1% *N*-acetyl-L-cysteine), shaken for 20 min at 800 rpm, and cultured on blood, chocolate, Endo, Sabouraud, and MacConkey agars. The last 53 samples were also cultured on the selective *B. cepacia* agar (Oxoid, Basingstoke, United Kingdom) that came into use during the study. All of the BCC isolates were identified by the API 20 NE system (Biomerieux, Marcy l'Etoile, France) in accordance with the supplier's protocol. For PCR analysis, sputum was mixed with an equal volume of the homogenization solution and shaken for 1 h at 800 rpm. DNA was extracted from 100 µl of liquefied sputum with the AMPLICOR Respiratory Specimen Preparation Kit (Roche, Indianapolis, Ind.) in accordance with the manufacturer's instructions. To check the DNA content in extraction aliquots, real-time PCR quantitation of the human albumin gene as an equivalent of the human DNA content was done on an ABI 7700 system (Applied Biosystems, Foster City, Calif.). Samples with albumin gene quantities below the fifth percentile of the first 100 assays were re-extracted.

To detect the open reading frame of the BCC *recA* gene, DNA extracts were subjected to nested PCRs. For the first PCR round, the *Taq* PCR Core Kit (Qiagen, Hilden, Germany) was used. The PCRs were carried out in duplicate in a total volume of 20 µl with 1× PCR buffer, 1× solution Q (Qiagen, Hilden, Germany), 2 mM MgCl₂, 200 µM (each) deoxynucleoside triphosphate, 14 pmol (each) of primers BCR1 and BCR2 (Table 1), 1 U of *Taq* polymerase, and 1 µl of the DNA extract. The PCR program was run on a GeneAmp 9700 thermocycler (Applied Biosystems, Foster City, Calif.): initial denaturation for 2 min at 94°C; 30 three-temperature cycles of 30 s at 94°C, 45 s at 62°C, and 90 s at 72°C; and a final extension of 5 min at 72°C. The second round of the nested PCR was performed with 1× PCR buffer (Promega), 2 mM MgCl₂, 200 µM (each) deoxynucleoside triphosphate, 10 pmol (each) of inner *recA* primers REC-IN-5 and BCRBM2 (Table 1), 0.5 U of *Taq* polymerase (Promega, Madison, Wis.), and 0.5 µl of the first-round PCR product. The amplification profile consisted of 25 three-temperature cycles of 30 s (for denaturation) at 94°C; 45 s (for annealing) at 67°C (first 5 cycles), 65°C (next 5 cycles), and 63°C (remaining 15 cycles); and 60 s (for synthesis) at 72°C. A final extension step of 5 min

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TABLE 1. Primers used in this study

Organism(s) detected	Primer mixture	5' primer name	Sequence (5'-3')	3' primer name	Sequence (5'-3')	Approximate product size (bp)
BCC (first run of nested PCR)	C-0	BCR1 ^a	TGACCGCCGAGAAGAGCAA	BCR2 ^a	CTCTTCTTCGTCCATCGCCTC	1040
BCC (second run of nested PCR)	C-IN	REC-IN-5 ^c	CATGATCGTCATCGACTCGGTC	BCRBM2 ^b	TCCATCGCCTCGGCTTCGT	620
Genomovar I	C-1	BCRG11 ^a	CAGGTCGTCTCCACGGGT	BCRG12 ^a	CACGCCGATCTTCATACGA	490
<i>B. multivorans</i>	C-2	BCRBM1 ^a	CGGCGTCAACGTGCCGGAT	C2-3 ^c	CTCGGCTTCGTCTGTGGC	710
Genomovar IIIA	C-3A	BCRG3A1 ^a	GCTCGACGTTCAATATGCC	BCRG3A2 ^a	TCGAGACGCACCGACGAG	380
Genomovar IIIB	C-3B	BCRG3B1 ^a	GCTGCAAGTCATCGCTGAA	BCRG3B2 ^a	TACGCCATCGGGCATGCT	780
<i>B. stabilis</i>	C-4	BCRG41 ^a	ACCGGCGAGCAGGCGCTT	BCRG42 ^a	ACGCCATCGGGCATGGCA	650
<i>B. vietnamiensis</i>	C-5	BCRBV1 ^a	GGGCGACGGCGACGTGAA	BCRBV2 ^a	TCGGCCTTCGGCACCAGT	380
Genomovar VI	C-6	BCRBM1 ^b	CGGCGTCAACGTGCCGGAT	C6-3 ^c	TGATGAAGATCACGAGGCAA	260
<i>B. ambifaria</i>	C-7	BCR1 ^b	TGACCGCCGAGAAGAGCAA	CA-3 ^c	CCTCGGCTTCGTCTGTGA	1,030

^a Genomovar-specific primer designed by Mahenthiralingam et al. (13).

^b Genomovar-specific primer designed by Mahenthiralingam et al. (13), now used in a sequence-specific primer PCR detecting genomovars other than those in the original paper.

^c Newly designed primer.

at 72°C followed. The PCR products of both rounds were run on a 2% agarose gel in 0.5% Tris-borate-EDTA buffer for 20 min at 10 V/cm.

In the BCC-positive samples, products of the first round were also used for genomovar status determination. The PCR product was diluted 1:10,000 (for samples positive in the first PCR round) or 1:100 (for samples that yielded a signal only in the second PCR round) with deionized, double-distilled water. The PCR setup was identical to that of the second round; the genomovar specificity of the eight reactions was maintained by the use of respective sequence-specific primers (Table 1).

New primer pairs were designed to identify *B. multivorans* (C-2), genomovar VI (C-6), and *B. ambifaria* (C-7). To check the sensitivity and specificity of the assays, we used strains from the BCC strain panel (14) completed with genomovar VI and *B. ambifaria* strains from the Laboratorium voor Microbiologie, Ghent, Belgium. The recently described species *B. pyrricinia* was not tested. Results are shown in Fig. 1. Each strain was correctly identified; moreover, our new 3' primers eliminated the previously described cross-reactivity between *B. multivorans* and genomovar VI (6). We also tested for reactivity with other bacteria possibly present in CF sputum (i.e., *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Haemophilus influenzae*, *Stenotrophomonas maltophilia*, *Alcaligenes xylosoxidans*, *Ralstonia pickettii*, *B. gladioli*, *Chryseobacterium meningosepticum*, and *Delftia acidovorans*). No reactivity was observed for either the generic C-IN primer mixture (used in the second round of the nested PCR) or the genomovar-specific primer pairs. To determine the sensitivity of the nested PCR, the procedure described by McDowell et al. (16) was applied to strains LMG 16656 (genomovar III-A) and LMG 13010 (*B. multivorans*). The detection limit of the nested PCR was 10³ CFU/ml of sputum.

Two hundred eleven sputum samples from 134 CF patients were tested. By culture techniques, the BCC organisms were recovered from 89 (42%) of 211 samples. Comparing results of growth on the nonselective agars and selective *B. cepacia* medium, we found no difference in the number of BCC organisms recovered. By the nested-PCR protocol, 110 samples (52%) were determined to be BCC positive, of which 28 yielded a

signal only in the second PCR round. Of the 134 patients examined, the BCC was detected in 49 patients by culture whereas it was detected in 67 patients by PCR. Thus, 18 more CF patients were found to be BCC positive by PCR than by cultivation.

A nested or seminested design is known to increase PCR sensitivity. The improvement in sensitivity over the nonnested setup was evident in 28 positive samples that were found to be positive only after the second round of PCR. This represents 25% of the 110 positive samples. Similarly, the superiority of two PCR rounds was demonstrated by Moore et al. (18), who employed a seminested approach for detection of *B. multivorans* and genomovar III. Although the sensitivity of the reported seminested strategy was 1 order of magnitude greater than the sensitivity of the present method, our protocol offers the sorting of BCC organisms into seven genomovars. To further minimize the risk of false negativity, we checked the yields of DNA extraction by using real-time quantification of human genome equivalents per microliter of DNA. If human DNA was not present in a sufficient quantity in the extract, failure of DNA extraction was highly probable since sputum contains large quantities of leukocytes.

As regards the genomovar identification of BCC organisms, 62 patients (92.5%) were colonized by genomovar III, *recA* group III-A; 3 were colonized by genomovar III, *recA* group III-B; and 2 were colonized by *B. multivorans*. None of the patients was infected by two or more genomovars, and replacement of one genomovar by another was not observed during the study period. In addition to analysis of clinical samples, we examined 44 BCC isolates recovered from sputum samples of 36 Czech CF patients collected from 1997 to 1999 and archived at the National Institute of Public Health, Prague, Czech Republic. All of them were identified as genomovar III, *recA* group III-A, which has been previously associated with the most severe course of the infection caused by the BCC (7, 12). The almost absolute uniformity of the BCC genomovar spectrum in the Czech CF community is alarming, and the identical finding among the archived samples suggests a possible epidemic origin of the infections. However, to fully explain this

FIG. 1. Genomovar-specific reactions on BCC reference strains ATCC 25416 (A), LMG 16660 (B), LMG 16656 (C), LMG 14294 (E), LMG 10929 (F), LMG 18941 (G), and LMG 19182 (H) and a clinical sample of genomovar III-B (D). The lanes contained sequence-specific primer PCR products of the genomovars. Lanes: 1, genomovar I; 2, *B. multivorans*; 3a, genomovar III-A; 3b, genomovar III-B; 4, *B. stabilis*; 5, *B. vietnamiensis*; 6, genomovar VI; 7, *B. ambifaria*; IN, generic detection of the BCC species. Molecular size markers (100-bp ladder) were run in lane M.

unfavorable phenomenon, further studies based on molecular typing have to be performed.

In conclusion, the protocol described herein offers rapid PCR detection of BCC in sputum and identification of its

genomovars. Previous assays for direct PCR detection of the BCC in sputum were based on amplification of the 16S rRNA gene (4, 8) or of the 16S-23S spacer region of the rRNA operon (22). Recently, McDowell et al. described PCR of the *recA* gene followed by restriction fragment length polymorphism (RFLP) analysis (16), concluding that the *recA* gene is a more suitable target than the 16S region. However, the PCR-RFLP assay can yield many different RFLP patterns, demanding attentive comparison (17). On the contrary, the algorithm presented here with nested PCR and genomovar-specific *recA* primers achieves high sensitivity and specificity with simple interpretation of genomovar results.

We are grateful to E. Mahenthiralingam for helpful advice and to P. Vandamme for providing the reference strains.

This work was supported by the Ministry of Health (projects NM/6568-3 and 64203) and the Ministry of Education (grant 111300003), Czech Republic.

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