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

Pavel Drvínek,1* Hana Hrbáčková,2 Ondřej Cinc,1 Jana Bartošová,1 Otakar Nyč,2 Alexandr Nemec,3 and Petr Pohněk1

2nd Department of Pediatrics, 2nd Medical School of Charles University,1 Department of Medical Microbiology, Motol University Hospital,2 and National Institute of Public Health,3 Prague, Czech Republic

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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. pyrrocinia* (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, specimens 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 (Bioriemerix, 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 *Tag* 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 *Tag* 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 *Tag* 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
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 genovar 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 two PCR rounds was demonstrated by Moore et al. (18), who employed a seminested approach for detection of *B. multivorans* and genovar 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 genovars. 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 genovar identification of BCC organisms, 62 patients (92.5%) were colonized by genovar III, recA group III-A; 3 were colonized by genovar III, recA group III-B; and 2 were colonized by *B. multivorans*. None of the patients was infected by two or more genovars, and replacement of one genovar 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 genovar 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 genovar 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

### 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<sup>a</sup> | TGAGCGCGAGAGAGACCA | BCR2<sup>a</sup> | CTCTTCCTCCTACATTCGGT | 1040 |
| BCC (second run of nested PCR) | C-IN | REC-IN<sup>5</sup> | CATGATCGTCTATCAGCTCGGAG | BCRBM1<sup>a</sup> | TCCATCGGCTCGGCTTCGT | 620 |
| *B. multivorans* | C-1 | BCRG11<sup>c</sup> | CAGTTCGCTTCACCCCGGGT | BCRG12<sup>c</sup> | CACCGGGAATCTTCATACGG | 490 |
| Genovar IIA | C-2 | BCRBM1<sup>a</sup> | CCGCGTGACCGTGCTCGGAG | C2-3<sup>c</sup> | CTGGCTCACGCTGCCTG | 710 |
| Genovar IIIIB | C-3A | BCRG3A1<sup>c</sup> | GTCCAGCTGCTCATCGTGA | BCRG2A<sup>c</sup> | TGGACGACGGGACGCAG | 380 |
| Genovar IIB | C-3B | BCRG3B1<sup>c</sup> | GGTGCGGTTGTTTGGGGAG | BCRG2B<sup>c</sup> | TGGCCCATGGGCGATC | 780 |
| *B. stabili* | C-4 | BCRG41<sup>c</sup> | ACACGGGCGAGCCGGCCTT | BCRG42<sup>c</sup> | AGCCGATGGGGGGATGC | 650 |
| *B. vietnamiensis* | C-5 | BCRBV1<sup>c</sup> | GGGCGGACGCGGACGGTAGA | BCRBV2<sup>c</sup> | TGCGCCGCTGGGACAGG | 380 |
| Genovar VI | C-6 | BCRBM1<sup>b</sup> | CGCGGTCAAGCAGGCGGA | C6-3<sup>c</sup> | TATGGAAGATCAGCAGGCA | 260 |
| *B. ambifaria* | C-7 | BCR1<sup>c</sup> | TGACCGCGAGAGAGACCA | CA-3<sup>c</sup> | CCTCGGCTTCGTCTGG | 1,080 |

<sup>a</sup>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 genovar 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 genovars. 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.

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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.

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REFERENCES

1. Aaron, S. D., W. Ferris, D. A. Henry, D. P. Speert, and N. E. Macdonald. 2000. Multiple combination bactericidal antibiotic testing for patients with cystic fibrosis infected with Burkholderia cepacia. Am. J. Respir. Crit. Care Med. 161:1206–1212.
2. Agodi, A., E. Mahenthiralingam, M. Barchitta, V. Giannina, A. Sciaccia, and S. Stefani. 2001. Burkholderia cepacia complex infection in Italian patients with cystic fibrosis: prevalence, epidemiology, and genomovar status. J. Clin. Microbiol. 39:2891–2896.
3. Beringer, P. M., and M. D. Appleman. 2000. Unusual respiratory bacterial flora in cystic fibrosis: microbiologic and clinical features. Curr. Opin. Pulm. Med. 6:545–550.
4. Campbell, P. W., J. A. Phillips, G. J. Heidecker, M. R. Krishnamani, R. Zaborchak, and T. L. Stull. 1995. Detection of Pseudomonas (Burkholderia) cepacia using PCR. Pediatr. Pulmonol. 20:44–49.
5. Coenye, T., P. Vandamme, J. R. Govan, and J. J. LiPuma. 2001. Taxonomy and identification of the Burkholderia cepacia complex. J. Clin. Microbiol. 39:3427–3434.
6. Henry, D. A., E. Mahenthiralingam, P. Vandamme, T. Coenye, and D. P. Speert. 2001. Phenotypic methods for determining genomovar status of the Burkholderia cepacia complex. J. Clin. Microbiol. 39:1073–1078.
7. Jones, A. M., M. E. Dodd, and A. K. Webb. 1996. Polymerase chain reaction for the detection of Pseudomonas aeruginosa, Stenotrophomonas maltophilia and Burkholderia cepacia in sputum of patients with cystic fibrosis. Mol. Cell. Probes 10:397–403.
8. Karpati, F., and J. Jonasson. 1996. Polymerase chain reaction for the detection of Pseudomonas aeruginosa, Stenotrophomonas maltophilia and Burkholderia cepacia in sputum of patients with cystic fibrosis. Mol. Cell. Probes 10:397–403.
9. LiPuma, J. J. 1998. Burkholderia cepacia. Management issues and new insights. Clin. Chest Med. 19:473–486.
10. LiPuma, J. J., B. J. Dulaney, J. D. McMenamin, P. W. Whitby, T. L. Stull, T. Coenye, and P. Vandamme. 1999. Development of rRNA-based PCR assays for identification of Burkholderia cepacia complex isolates recovered from cystic fibrosis patients. J. Clin. Microbiol. 37:3167–3170.
11. LiPuma, J. J., T. Spilker, L. H. Gill, P. W. Campbell III, L. Liu, and E. Mahenthiralingam. 2001. Diportportionate distribution of Burkholderia cepacia complex species and transmissibility markers in cystic fibrosis. Am. J. Respir. Crit. Care Med. 164:92–96.
12. Mahenthiralingam, E., J. Bischof, S. K. Byrne, C. Radomski, J. E. Davies, Y. Av-Gay, and P. Vandamme. 2000. DNA-based diagnostic approaches for identification of Burkholderia cepacia complex, Burkholderia vietnamiensis, Burkholderia stabilis, and Burkholderia cepacia genomovars I and III. J. Clin. Microbiol. 38:3165–3173.
13. Mahenthiralingam, E., T. Coenye, J. W. Chung, D. P. Speert, J. R. Govan, P. Taylor, and P. Vandamme. 2000. Diagnostically and experimentally useful panel of strains from the Burkholderia cepacia complex. J. Clin. Microbiol. 38:910–913.
14. Mahenthiralingam, E., P. Vandamme, M. E. Campbell, D. A. Henry, A. M. Gravelle, L. T. Wong, A. G. Davidson, P. G. Wilcox, B. Nakielna, and D. P. Speert. 2001. Infection with Burkholderia cepacia complex genomovars in patients with cystic fibrosis: virulent transmissible strains of genomovar III can replace Burkholderia multivorans. Clin. Infect. Dis. 33:1469–1475.
Elborn, 2001. PCR-based detection and identification of Burkholderia cepacia complex pathogens in sputum from cystic fibrosis patients. J. Clin. Microbiol. 39:4247–4255.

17. Moore, J. E., B. C. Millar, J. Xu, M. Crowe, A. O. Redmond, and J. S. Elborn, 2002. Misidentification of a genomovar of Burkholderia cepacia by recA restriction fragment length polymorphism. J. Clin. Pathol. 55:309–311.

18. Moore, J. E., J. Xu, B. C. Millar, M. Crowe, and J. S. Elborn. 2002. Improved molecular detection of Burkholderia cepacia genomovar III and Burkholderia multivorans directly from sputum of patients with cystic fibrosis. J. Microbiol. Methods 49:183–191.

19. O’Callaghan, E. M., M. S. Tanner, and G. J. Boulnois. 1994. Development of a PCR probe test for identifying Pseudomonas aeruginosa and Pseudomonas (Burkholderia) cepacia. J. Clin. Pathol. 47:222–226.

20. Segonds, C., T. Heulin, N. Marty, and G. Chabanon. 1999. Differentiation of Burkholderia species by PCR-restriction fragment length polymorphism analysis of the 16S rRNA gene and application to cystic fibrosis isolates. J. Clin. Microbiol. 37:2201–2208.

21. van Pelt, C., C. M. Verduin, W. H. Goessens, M. C. Vos, B. Tummler, C. Segonds, F. Reuhsaet, H. Verbrugh, and A. van Belkum. 1999. Identification of Burkholderia spp. in the clinical microbiology laboratory: comparison of conventional and molecular methods. J. Clin. Microbiol. 37:2158–2164.

22. Whitby, P. W., H. L. Dick, P. W. Campbell, 3rd, D. E. Tullis, A. Matlow, and T. L. Stull. 1998. Comparison of culture and PCR for detection of Burkholderia cepacia in sputum samples of patients with cystic fibrosis. J. Clin. Microbiol. 36:1642–1645.
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Characterization of Multidrug-Resistant Escherichia coli Isolates Associated with Nosocomial Infections in Dogs
Susan Sanchez, M. A. McCrackin Stevenson, Charlene R. Hudson, Marie Maier, Tameka Buffington, Quyen Dam, and John J. Maurer

Athens Diagnostic Laboratory and the Departments of Medical Microbiology and Parasitology, Small Animal Medicine, and Avian Medicine, College of Veterinary Medicine, The University of Georgia, and Agricultural Research Service, U.S. Department of Agriculture, Russell Research Center, Athens, Georgia 30602

Volume 40, no. 10, p. 3586–3595, 2002. Page 3589, Table 2, column 8: Line 17 should read “280, 115” and line 18 should be blank.

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2nd Department of Pediatrics, 2nd Medical School of Charles University,1 Department of Medical Microbiology, Motol University Hospital,2 and National Institute of Public Health,3 Prague, Czech Republic

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