Identification of *Burkholderia cepacia* complex pathogens by rapid-cycle PCR with fluorescent hybridization probes

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Members of the *Burkholderia cepacia* complex are important bacterial pathogens in cystic fibrosis (CF) patients. The *B. cepacia* complex currently consists of nine genetic subgroups (genomovars) of different epidemiological relevance and possibly of different pathogenic potential in humans. In this study, a new approach was developed for the rapid identification of *B. cepacia* genomovar I, *Burkholderia multivorans* (genomovar II), *Burkholderia cenocepacia* (lineage III-A and III-B), *Burkholderia stabilis* (genomovar IV) and *Burkholderia vietnamiensis* (genomovar V), which cause the large majority of infections in CF patients. The method was based on the detection of differences in the *recA* gene sequence by using rapid-cycle PCR and genomovar-specific fluorescence resonance energy transfer (FRET) probes. The genomovar status of all 39 *B. cepacia* complex strains tested (genomovars I–V) was identified by melting-curve analysis. Each FRET probe produced a specific fluorescence signal only with the respective genomovar, and not with other *B. cepacia* complex strains and *Burkholderia* spp. The identification system was easy to handle and revealed *B. cepacia* complex genomovar I–V status from culture isolates within about 1 h.

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INTRODUCTION

Burkholderia cepacia, first described by Burkholder as the cause of soft rot in onions, is commonly found throughout the environment and causes opportunistic infections in humans. During the last few years, it has been recognized that organisms previously identified as *B. cepacia* appear to be very heterogeneous and constitute a complex of phenotypically similar species, among which at least nine *Burkholderia* species can be differentiated by molecular methods. In patients with cystic fibrosis (CF), *B. cepacia* complex strains are associated with the ‘cepacia syndrome’, a necrotizing pneumonia with bacteraemia, which is often fatal (Isles *et al.*, 1984). The use of *B. cepacia* complex strains as a fertilizer and pesticide may serve as a source of infection for patients, although there has been no proof so far that

agriculturally used strains lead to infections of CF patients (Balandreau *et al.*, 2001; LiPuma *et al.*, 2002). Person-to-person transmission of *B. cepacia* complex strains through social contact has been demonstrated (Agodi *et al.*, 2002; Govan *et al.*, 1993), leading to attempts to reduce this risk by segregation of patients (Thomassen *et al.*, 1985; Muhdi *et al.*, 1996). Due to the poor prognosis after allogenic lung transplantation (Snell *et al.*, 1993; Chaparro *et al.*, 2001), some transplant centres have excluded patients colonized with *B. cepacia* complex strains from lung transplantation programmes (Aris *et al.*, 2001).

So far, *B. cepacia* genomovar I, *Burkholderia multivorans* (genomovar II) (Vandamme *et al.*, 1997), *Burkholderia cenocepacia* (genomovar III) (Vandamme *et al.*, 2003), *Burkholderia stabilis* (genomovar IV) (Vandamme *et al.*, 2000), *Burkholderia vietnamiensis* (genomovar V) (Gillis *et al.*, 1995), *Burkholderia dolosa* (genomovar VI) (Vermis *et al.*, 2004; Coenye *et al.*, 2001a), *Burkholderia ambifaria* (genomovar VII) (Coenye *et al.*, 2001b), *Burkholderia anthina* (genomovar VIII) and *Burkholderia pyrrocinia* (genomovar

Abbreviations: CF, cystic fibrosis; FRET, fluorescence resonance energy transfer.

The strains used in this study are listed in Supplementary Table S1 with the online version of this paper.

IX) (Coenye *et al.*, 2001c) have been identified within the *B. cepacia* complex. Epidemiological studies have indicated differences in transmission frequency and pathogenicity within the CF population depending upon genomovar status (Biddick *et al.*, 2003). In particular, *B. cenocepacia* seems to be associated with high transmission frequency and poor prognosis (Woods *et al.*, 2004).

Several methods have been used to identify the genomovar status of members of the *B. cepacia* complex. Phenotypic tests reliably separate only *B. multivorans* and *B. stabilis* (Henry *et al.*, 2001). 23S rDNA- and 16S rDNA-based PCR assays allow the differentiation of *B. multivorans* and *B. vietnamiensis*, but not *B. cepacia*, *B. cenocepacia* or *B. stabilis* (LiPuma *et al.*, 1999; Bauernfeind *et al.*, 1999). Whitby *et al.* (2000) have developed a PCR algorithm of the 16S–23S rDNA spacer region that is able to separate *B. cenocepacia* and *B. stabilis*, but not *B. cepacia*. Conventional amplified fragment length polymorphism (AFLP) analysis can be used for *B. cepacia* complex differentiation, but is technically demanding, while automated AFLP still requires visual examination for correct identification of all isolates (Coenye *et al.*, 1999). RFLP of 16S rDNA leads to the identification of groups, but is unable to distinguish many strains of *B. cepacia*, *B. cenocepacia*, *B. stabilis* and *B. pyrrocinia* (Segonds *et al.*, 1999; Vermis *et al.*, 2002b). Discrimination of *B. cepacia* genomovar I, *B. multivorans*, *B. cepacia* genomovar III, *B. stabilis*, *B. vietnamiensis* and *B. ambifaria* is possible by RFLP analysis of the PCR-amplified *recA* gene using *B. cepacia* complex-specific *recA* primers (Mahenthiralingam *et al.*, 2000; Coenye *et al.*, 2001c). This approach has also been used by McDowell *et al.* (2001) for direct detection of *B. cepacia* complex strains in sputum of CF patients. Most *B. cepacia* complex genomovars can be identified by using *recA*-based genomovar-specific primers (Mahenthiralingam *et al.*, 2000). Based on this approach, nested PCR assays have been described that improve the sensitivity of *B. cepacia* complex detection from sputum specimens (Moore *et al.*,

2002; Drevinek *et al.*, 2002). A recent evaluation of genomovar-specific *recA*-based PCR tests by Vermis *et al.* (2002a) has revealed that PCR primers designed to be specific for *B. cepacia* genomovar I cross-react with *B. pyrrocinia* and fail to detect some genomovar I isolates. Since the rapid and reliable detection of *B. cepacia* complex organisms is important for infection control measures as well as for the clinical management of patients, a rapid method of genomovar determination suitable for daily use in the clinical laboratory is desirable.

The aim of this study was to develop a rapid test based on LightCycler technology for the differentiation of the most common *B. cepacia* complex culture isolates, based on the known polymorphism of the *recA* gene (Mahenthiralingam *et al.*, 2000). The LightCycler technique is based on conventional PCR, but reduces the DNA amplification time by increasing the temperature transition rate through the use of air for heating and cooling (Cockerill & Smith, 2002). Mutations within genes can be detected by sequence-specific fluorescence resonance energy transfer (FRET) probes labelled with two different fluorescence dyes that generate a fluorescence signal only when both probes bind to the target sequence. After the amplification process, a melting-curve analysis is performed: the fluorescence is monitored with increasing temperature. A decrease in fluorescence is obtained when probes melt off. Mismatches between probe sequence and target sequence lead to a lowered melting temperature.

B. cepacia, *B. multivorans*, *B. cenocepacia*, *B. stabilis* and *B. vietnamiensis* usually comprise more than 95 % of *B. cepacia* complex isolates in CF patients (Speert *et al.*, 2002; Agodi *et al.*, 2001; LiPuma *et al.*, 2001). We therefore developed an identification protocol based on *B. cepacia* complex-specific LightCycler amplification of the *recA* locus and newly designed genomovar-specific FRET probes for the rapid detection of genomovars I–V.

Table 1. Sequences of genomovar-specific FRET probes

Probe	Sequence (5'–3')	Orientation	Position (bp)
I – anchor	LightCycler Red 640-CCG AGC TTC GAC GCG TAC TGG A	Antisense	305–272
I – sensor	GCA GTT CCG GCA CAT TCA C-fluorescein	Antisense	329–346
II – anchor	LightCycler Red 640-GTC CAC GGG CTC GCT CGG	Sense	120–138
II – sensor	GCC GAG GAC ATC CAG GTC G-fluorescein	Sense	100–118
III-A – anchor	LightCycler Red 640-CGT GAA CGT GCC GGA GCT GC	Sense	327–346
III-A – sensor	CGT TCA ATA TGC CGC GAA GCT C-fluorescein	Sense	303–324
III-B – anchor	LightCycler Red 705-AAG ATC GGC GGC ACG GCA G	Sense	257–274
III-B – sensor	CTG CAA GTC ATC GCT GAA CTG C-fluorescein	Sense	232–253
IV – anchor	TGA TCT CGC AGC CGG ACA CCG-fluorescein	Sense	347–367
IV – sensor	LightCycler Red 705-CGA GCA GGC GCT TGA AAT CAC	Sense	369–389
V – anchor	LightCycler Red 705-AGG TCG TCT CCA CGG GCT CGC T	Sense	114–134
V – sensor	GGC GAC GTG AAG GAA GAC ATC-fluorescein	Sense	91–111

METHODS

Bacterial strains and template preparation. Strains of *B. cepacia* genomovar I, *B. multivorans* (II), *B. cenocepacia* lineages III-A and III-B, *B. stabilis* (IV) and *B. vietnamiensis* (V) were received from the Belgian Coordinated Collections of Microorganisms/Laboratorium voor Microbiologie Universiteit Gent (BCCM/LMG) Bacteria Collection or were clinical isolates of Hannover Medical School, Hannover, Germany, which were tentatively identified as *B. cepacia* after growth on OFBL agar (Henry *et al.*, 1997) and determination of biochemical profiles using API 20NE. These strains were further identified by SDS-PAGE of whole-cell proteins and, if necessary, by *recA* RFLP as described by Coenye *et al.* (2001b). All strains, including non-*B. cepacia* complex organisms used in this study, are listed in Supplementary Table S1 with the online version of this paper.

Bacteria were grown overnight on Columbia blood agar plates at 37 °C. Ten colonies were then mixed with 1 µl DNA-free water. Two microlitres of this suspension served as template. No further DNA extraction was performed. In addition to non-*B. cepacia* complex bacterial suspensions, DNA-free water also served as a negative control.

LightCycler protocol. A LightCycler system (Roche Molecular Biochemicals) and FRET probes (TIB MOLBIOL) labelled with different LightCycler Red dyes (640 and 705 nm) were used. FRET probes were designed to show a perfect match only to the corresponding genomovar. When FRET probes bind to their target sequence, a fluorescence signal is detected by the system. If denaturation occurs, a loss of fluorescence is observed. Therefore, if the target sequence shows a mismatch, the melting temperature of FRET

Fig. 1. *B. cepacia* complex genomovar identification using genomovar-specific FRET probes. Screen captures of melting-peak analyses are shown. The melting-curve analysis is displayed as the first negative derivative of the fluorescence ($-dF/dT$) versus temperature. F2 refers to channel 2, which was used to measure signals from LightCycler Red 640 at 640 nm (A–C). F3 refers to channel 3, which measures signals from LightCycler Red 705 at 705 nm (D–F). The identification with the specific FRET probes of representative strains of *B. cepacia* (five strains) (A), *B. multivorans* (four strains) (B), *B. cenocepacia* lineage III-A (four strains) (C), *B. cenocepacia* lineage III-B (four strains) (D), *B. stabilis* (five strains) (E) and *B. vietnamiensis* (five strains) (F) is shown. *B. dolosa* (genomovar VI) (brown), *B. ambifaria* (genomovar VII) (purple), *B. anthina* (genomovar VIII) (grey) and *B. pyrrocinia* (genomovar IX) (pink), and DNA-free water (black), served as controls. Detection probes showed the highest melting temperature with representative strains of the corresponding genomovar, while other genomovars showed lower non-specific melting peaks or no peaks at all.

probes is lowered. Visualization of the melting temperature at which the probe melted from the amplicon from the loss of fluorescence that occurred was done by plotting $-d(F2)/dT$ against temperature.

Amplification of the *B. cepacia* complex *recA* gene was performed using a total volume of 18 μ l of the ready-to-use reaction mixture of the Fast Start DNA Master Hybridization Probe kit (Roche Diagnostics), supplemented with 4 mM $MgCl_2$, 10 pM of each primer BCR1 and BCR2 (MWG-Biotech) specific for the *B. cepacia* complex *recA* gene, as described by Mahenthalingam *et al.* (2000) (500 nM final concentration), and 2 pM of two probe pairs for genomovar determination (100 nM final concentration). Two microlitres of template as described above were added for a total sample volume of 20 μ l in each glass capillary.

After an initial denaturation step (95 °C, 10 min), 45 cycles consisting of denaturation (95 °C, 10 s each), annealing (reached with a

touch-down from 66 to 60 °C at 1 °C per cycle, 10 s each) and elongation (72 °C, 20 s) were run for *recA* gene amplification. After a final denaturation (95 °C, 30 s), melting-curve analysis from 40 up to 75 °C was done, measuring fluorescence at 640 and 705 nm continuously. The temperature transition rate was 20 °C s^{-1} for all changes in temperature, except for melting-curve analysis, for which the rate was lowered to 0.1 °C s^{-1} .

The sequences of FRET probes used for genomovar determination are listed in Table 1. Probe pairs for detection of genomovars I, II and III-A were labelled with LightCycler Red 640, and probe pairs for genomovars III-B, IV and V were labelled with LightCycler Red 705, respectively. Colour compensation software was used when FRET probes with labels of different wavelengths were tested simultaneously. The sequences of hybridization probes were based on the alignment of published *recA* sequences of defined *B. cepacia* complex genomovars

I–IX (GenBank accession nos AF143774–AF143795, AF143798–AF143799, AF323985, AF456003, AF456005–AF456007, AF456012–AF456014, AF456021, AF456027–AF456033, AF456045, AF456055, AF456058, AF456060, AF456070, AF456076–AF456077, AF456093, AF456097, AF456102, AF456104, AF456106–AF456108 and AJ544692) using DNA Star Windows 32 MegAlign 4.00 software.

To determine the sensitivity of our LightCycler protocol, a template suspension was prepared as described above. Serial dilution of the template suspension using DNA-free water was performed. Diluted probes then were used for LightCycler detection. In addition, diluted probes were used to calibrate LightCycler detection against bacterial c.f.u. after growth overnight on Columbia blood agar plates at 37 °C.

RESULTS AND DISCUSSION

We first tested amplification of the *recA* gene with primers BCR1 and BCR2 (Mahenthiralingam *et al.*, 2000; McDowell *et al.*, 2001) in strains belonging to *B. cepacia* complex genomovars I, II, III-A, III-B, IV and V using LightCycler technology. Under these experimental conditions, primers BCR1 and BCR2 proved to be specific for all these genomovars, as described for conventional PCR (Mahenthiralingam *et al.*, 2000), and led to a PCR product of 1043 bp visualized by conventional gel electrophoresis, whereas non-*B. cepacia* complex members did not show an amplification product of this size (not shown).

After alignment of *recA* sequences from genomovar I to genomovar IX, genomovar-specific portions were used to design FRET probes. Sequences are shown in Table 1. Using these probes, the genomovar status of all 39 *B. cepacia* complex strains belonging to genomovars I, II, III-A, III-B, IV and V was correctly identified by melting-curve analysis. Fig. 1 shows the specific peak loss of fluorescence of the detection probes of four representative strains of *B.*

multivorans (Fig. 1B), *B. cenocepacia* III-A (Fig. 1C) and *B. cenocepacia* III-B (Fig. 1D), and of five strains of *B. cepacia* (Fig. 1A), *B. stabilis* (Fig. 1E) and *B. vietnamiensis* (Fig. 1F). Each probe pair was tested against members of all other genomovars and lineages as well as against the non-*B. cepacia* complex strains listed above, in order to exclude possible interference. For all other genomovars, including lineages III-C and III-D, only non-specific melting points at significantly lower temperatures, or no peaks at all, were observed (data not shown).

The observed melting-point differences between strains within one genomovar (Fig. 1) are most likely due to point mutations in the portion of the *recA* sequence used for hybridization. For example, differences of peak melting point between the *B. cepacia* strains LMG 1222^T (GenBank accession no. AF143786) and strains LMG 18821 and LMG 2161 (GenBank accession nos AF143787 and AF143788), as shown in Fig. 1(A), are due to a single nucleotide mismatch at position 339. We observed a similar phenomenon for *B. cenocepacia* III-B (Fig. 1D) and *B. vietnamiensis* (Fig. 1F). However, the differences in the results of the melting-curve analysis were still sufficiently large to distinguish these genomovars from all other genomovars. Our LightCycler method could detect as few as 5 c.f.u. of all genomovar I–V strains tested. Fig. 2 shows a representative assay for *B. cepacia* lineage III-B LMG 16659.

In conclusion, we were able to develop a rapid-cycle PCR system that allowed determination of *B. cepacia* complex genomovars I–V from culture isolates within about 1 h using genomovar-specific FRET probes. This approach could also discriminate genomovar I from *B. pyrrocinia* (genomovar IX), since there was no melting-point signal

Fig. 2. Sensitivity of *B. cepacia* complex genomovar identification. Screen captures of melting-peak analyses and gel electrophoresis of amplicons of a representative strain (*B. cenocepacia* lineage III-B LMG 16659) are shown. Five c.f.u. of *B. cepacia* complex strains were reproducibly detected in genomovars I–V. Key: 0, 1 kb DNA ladder; 1, 100 c.f.u.; 2, 50 c.f.u.; 3, 10 c.f.u.; 4, 5 c.f.u.; 5, no template.

with a genomovar I-specific FRET probe and *B. pyrocinia*, as shown in Fig. 1(A). This discrimination is not possible by conventional genomovar-specific PCR (Vermis *et al.*, 2002a). Our method is based on the use of commercially available components under standardized conditions. It should therefore enable any clinical laboratory with access to LightCycler technology to distinguish between these genomovars, which exhibit different transmission frequencies and probably different pathogenic potential (Agodi *et al.*, 2001; Speert *et al.*, 2002; LiPuma *et al.*, 2001). Rapid-cycle real-time PCR has not only been used for the differentiation of a variety of pathogens from cultures but also for direct detection from clinical specimens. In first experiments, using the LightCycler technology, we have been able to detect directly genomovars I–V in CF sputum specimens seeded with the respective organisms (data not shown). Future studies will have to evaluate the sensitivity and specificity of this method for clinical specimens.

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