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.

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...
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; Bauerfeind 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 II, 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.

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Table 1. Sequences of genomovar-specific FRET probes

<table>
<thead>
<tr>
<th>Probes</th>
<th>Sequence (5’–3’)</th>
<th>Orientation</th>
<th>Position (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I – anchor</td>
<td>LightCycler Red 640-CGC AGC TTC GAC GCG TAC TGG A</td>
<td>Antisense</td>
<td>305–272</td>
</tr>
<tr>
<td>I – sensor</td>
<td>GCA GTT CCG GCA CAT TCA G-fluorescein</td>
<td>Antisense</td>
<td>329–346</td>
</tr>
<tr>
<td>II – anchor</td>
<td>LightCycler Red 640-GTC CAC GGG CTC GCT CGG</td>
<td>Sense</td>
<td>120–138</td>
</tr>
<tr>
<td>II – sensor</td>
<td>GCC GAG GAC ATC GTC G-fluorescein</td>
<td>Sense</td>
<td>100–118</td>
</tr>
<tr>
<td>III-A – anchor</td>
<td>LightCycler Red 640-CGT GAA CGT GGC GGA GCT GC</td>
<td>Sense</td>
<td>327–346</td>
</tr>
<tr>
<td>III-A – sensor</td>
<td>CGT TCA ATA TGC CGG GAA GCT C-fluorescein</td>
<td>Sense</td>
<td>303–324</td>
</tr>
<tr>
<td>III-B – anchor</td>
<td>LightCycler Red 705-AGG ATC GGC GGC ACG GCA G</td>
<td>Sense</td>
<td>257–274</td>
</tr>
<tr>
<td>III-B – sensor</td>
<td>CTG CAA GTC ATC GCT GAA CTG C-fluorescein</td>
<td>Sense</td>
<td>232–253</td>
</tr>
<tr>
<td>IV – anchor</td>
<td>TGA TCT CGC AGC CGG ACA CGG-fluorescein</td>
<td>Sense</td>
<td>347–367</td>
</tr>
<tr>
<td>IV – sensor</td>
<td>LightCycler Red 705-CGC GCA GCC GGC GTC GTA CAT GAC</td>
<td>Sense</td>
<td>369–389</td>
</tr>
<tr>
<td>V – anchor</td>
<td>LightCycler Red 705-AGG TCG TCT CCA CGG GCT GCT T</td>
<td>Sense</td>
<td>114–134</td>
</tr>
<tr>
<td>V – sensor</td>
<td>GGC GAC GTG AAG GAA GAC ATC-fluorescein</td>
<td>Sense</td>
<td>91–111</td>
</tr>
</tbody>
</table>
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 1μl 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 probes is lower.
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 $-\frac{dF}{dT}$ against temperature.

Amplification of the \textit{B. cepacia} complex \textit{recA} gene was performed using a total volume of 18 \textmu{}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 \textmu{}M of each primer BCR1 and BCR2 (MWG-Biotech) specific for the \textit{B. cepacia} complex \textit{recA} gene, as described by Mahenthiralingam \textit{et al.} (2000) (500 nM final concentration), and 2 \textmu{}M 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 \textmu{}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 \textit{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 \textit{recA} sequences of defined \textit{B. cepacia} complex genomovars.
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 1222T (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. pyrrocinia*, 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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**REFERENCES**


