MOLECULAR CHARACTERISATION

Evaluation of species-specific recA-based PCR tests for genomovar level identification within the Burkholderia cepacia complex

KAREN VERMIS, TOM COENYE*, ESHWAR MAHENTHIRALINGAM*, HANS J. NELIS and PETER VANDAMME*

Laboratory of Pharmaceutical Microbiology and *Laboratory of Microbiology, Ghent University, Ghent, Belgium and 1Cardiff School of Biosciences, Cardiff University, Cardiff

The Burkholderia cepacia complex presently comprises nine genomovars: 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), B. anthina (genomovar VIII) and B. pyrrocinia (genomovar IX). Strains of each genomovar can colonise the respiratory tract of cystic fibrosis (CF) patients. However, the majority of infections in CF patients are caused by B. multivorans and B. cepacia genomovar III isolates. Accurate genomovar-level identification is best achieved through a polyphasic approach combining phenotypic and genotypic analyses. In the present study, the sensitivity and specificity of recA-based genomovar specific primer pairs were evaluated with a collection of 508 B. cepacia complex isolates representing all nine genomovars. The assays for the identification of B. multivorans (sensitivity and specificity, 100%), B. cepacia genomovar III (sensitivity, 92%; specificity, 100%), and B. ambifaria (sensitivity and specificity, 100%) were the most efficient. However, the B. cepacia genomovar I assay lacked sensitivity (72%) and cross-reacted with all B. pyrrocinia isolates examined. Several new recA RFLP types were also revealed within the B. cepacia complex. One of these profiles was shared by a clinical and an environmental B. cepacia-like isolate and by the B. ubonensis type strain. The latter organism is a recently described soil bacterium. Its relationship to the various B. cepacia complex genomovars needs further study.

Introduction

The clinical significance of Burkholderia cepacia, originally described as a plant pathogen in the 1950s [1], increased in the 1980s as it emerged as an opportunist pathogen, colonising the lungs of cystic fibrosis (CF) patients [2–4] and immunocompromised individuals [5–7]. At its peak incidence in the mid-1980s, c. 20% of colonised patients suffered from severe progressive respiratory failure with bacteraemia (‘cepacia syndrome’) which was often fatal [2, 8]. Accumulated evidence of nosocomial acquisition and person-to-person transmission led to the implementation of stringent infection control measures, which resulted in a decline of new infections [9–11]. However, it was apparent that not all B. cepacia strains have the same capacity for epidemic cross-infection or are capable of causing fatal pneumonia [8, 12, 13]. Taxonomic studies have revealed that B. cepacia consists of a group of at least five phenotypically similar and closely related genomic species (B. cepacia genomovars I–V), which has become known as the B. cepacia complex [14]. Recently, four further taxa (B. cepacia genomovars VI–IX) have been described as members of the complex [15–17]. Discrimination between several of these genomovars is sometimes difficult. A binomial name was assigned to those genomovars that could be differentiated phenotypically, i.e., B. multivorans (formerly genomovar II) [14], B. stabilis (genomovar IV) [18], B. ambifaria (genomovar VII) [16] and B. anthina (genomovar VIII) [17]. Genomovar V and genomovar IX have been recognised as the previously described species B. vietnamiensis [19] and B. pyrrocinia, respectively [20]. B. cepacia genomovar III and B. cepacia genomovar VI cannot be
differentiated in biochemical tests from \textit{B. cepacia} genomovar I and \textit{B. multivorans}, respectively.

Currently, in our laboratories accurate identification of members of the \textit{B. cepacia} complex is the result of a polyphasic approach, including biochemical tests, whole-cell protein electrophoresis, DNA–DNA hybridisation and \textit{recA} restriction fragment length polymorphism (RFLP) analysis. A single test procedure to distinguish between all genomovars is not yet available. Molecular diagnostic tests based on species-specific rDNA PCR [21–23] and PCR-RFLP of 16S rDNA [24, 25] are useful to identify \textit{B. cepacia} genomovars II, IV, V and VI. However, \textit{B. cepacia} genomovars I, III and VII cannot be distinguished from each other. Moreover, there are no published data for \textit{B. anthina} and \textit{B. pyrrocinia} [24]. In recent studies, Mahenthiralingam and co-workers reported that \textit{recA} gene sequence comparison offered a superior potential for the development of genomovar-specific PCR tests [16, 26]. At present, specific primers are available for the detection of \textit{B. cepacia} genomovar I, \textit{B. multivorans}, \textit{B. cepacia} genomovars III-A and III-B (two distinct \textit{recA}-based phylogenetic subgroups within genomovar III), \textit{B. stabilis}, \textit{B. vietnamiensis} [26] and \textit{B. ambifaria} [16]. An error in one of the published primer sequences was corrected in an erratum available at http://ijs.sgmjournals.org/cgi/content/full/51/4/1481/DC1.

Although all \textit{B. cepacia} genomovars can colonise CF patients, the large majority of infections is caused by \textit{B. multivorans} or \textit{B. cepacia} genomovar III [12, 13, 27]. The aim of the present study was to evaluate the sensitivity and specificity of \textit{recA}-based PCR tests for the identification of these \textit{B. cepacia} genomovars and of PCR tests for \textit{B. cepacia} genomovar I and \textit{B. ambifaria}. The latter genomovars are very difficult to distinguish from \textit{B. cepacia} genomovar III. Whereas the original set of isolates used to design the \textit{recA}-based PCR tests and to evaluate their sensitivity and specificity was primarily of CF origin, the 508 isolates of the present study were obtained from a range of environmental sources and from human infections (CF and non-CF). They were isolated in 19 countries in Europe, North and South America, Asia and Australia.

\textbf{Materials and methods}

\textbf{Bacterial strains}

All isolates were grown aerobically at 28°C on Trypticase Soy Agar (Becton Dickinson, Sparks, MD, USA). Species or genomovar assignment was based on a combination of results obtained from whole-cell protein electrophoresis [14] and \textit{recA} RFLP analysis [26]. \textit{B. cepacia} genomovar I was represented by 29 clinical and 42 environmental isolates, \textit{B. multivorans} by 85 clinical and 17 environmental isolates, \textit{B. cepacia} genomovar III by 172 clinical and 62 environmental isolates, \textit{B. stabilis} and \textit{B. vietnamiensis} by 6 clinical and 1 environmental isolate each, \textit{B. cepacia} genomovar VI by 4 clinical isolates, \textit{B. ambifaria} by 6 clinical and 47 environmental isolates, \textit{B. anthina} by 5 clinical and 15 environmental isolates and \textit{B. pyrrocinia} by 7 environmental isolates. Two isolates (1 clinical and 1 environmental) with a \textit{recA} RFLP pattern different from the recognised patterns within the \textit{B. cepacia} complex were also included. The \textit{recA} RFLP profile of the latter isolates was identical to that of \textit{B. abonensis} LMG 20358T, a recently described soil bacterium [28], which was also included in the present study.

\textbf{DNA preparation and PCR assays}

DNA was prepared as described before [16]. PCR assays for the identification of \textit{B. cepacia} genomovars I and III, \textit{B. multivorans} and \textit{B. ambifaria} were performed as described previously [16, 26] with the following modifications. The annealing temperature for the PCR tests for \textit{B. multivorans} and \textit{B. cepacia} genomovars III-A and III-B was raised to 64°C, 68°C and 68°C, respectively, to eliminate non-specific primer binding. Furthermore, the correct primer sequence for primer BCRGC2 used for the identification of \textit{B. ambifaria} is 5'-TCC GCA GCC GCA CCT TCA-3'. The PCR apparatus used was a GeneAmp PCR System 9600 (Perkin-Elmer Applied Biosystems, Foster City, CA, USA).

\textit{recA} gene sequencing and RFLP analyses

\textit{recA} gene sequencing and RFLP analyses were performed as described before [26]. The nucleotide sequence of the entire \textit{recA} gene from the following strains was determined and deposited in GenBank (GenBank accession no. shown in brackets): LMG 14095 (AF456016), LMG 6863 (AF456019), LMG 18943 (AF456029), L06 (AF456030), LMG 14294 (AF456031), ATCC 39277 (AF456032), LMG 6964 (AF456065), LMG 6963 (AF456068), R-10741 (AF456084) and R-14268 (AF456122). These sequences were aligned by use of the CLUSTAL W algorithm with \textit{recA} sequences from \textit{B. cepacia} complex strains analysed in previous studies and a neighbour-joining genetic distance phylogenetic tree constructed as described previously [16, 18, 26].

\textbf{DNA–DNA hybridisation}

The methodology used for DNA–DNA hybridisation experiments (including DNA preparation) was as described previously [15]. The hybridisation temperature was 50°C.
Results and discussion

Each positive PCR test resulted in an amplification product of the expected size [16, 26].

Fifty-one of 71 isolates of *B. cepacia* genomovar I gave positive results with the genomovar I specific primers (sensitivity, 71.8%). No cross-reactions were observed with primers specific for other genomovars. All but one of the 20 remaining *B. cepacia* genomovar I isolates belonged to two new *recA* RFLP profiles within *B. cepacia* genomovar I. *RecA* sequence analysis confirmed that one of these types, designated AW, clusters close to previously characterised genomovar I *recA* sequences [26] (Fig. 1). The second type, designated K, occupied a distinct position in the phylogenetic tree. DNA–DNA hybridisation between two of these *recA* type K isolates (LMG 14095 and LMG 6863) and the *B. cepacia* genomovar I type strain (LMG 1222) revealed hybridisation values of 71% and 75%, respectively, confirming their identity as *B. cepacia* genomovar I.

Ninety-seven of 234 *B. cepacia* genomovar III isolates gave positive results in the *B. cepacia* genomovar III-A PCR test, and 114 *B. cepacia* genomovar III isolates reacted with the *B. cepacia* genomovar III-B primer pair (combined sensitivity for *B. cepacia* genomovar III, 92.2%). Five *B. cepacia* genomovar III isolates generated an amplicon with both the III-A and III-B specific primer pairs and 18 genomovar III strains failed to react with either of the genomovar III specific primer pairs. The *recA* RFLP patterns of these false-negative results were heterogeneous. Six strains were of *recA* RFLP type H, five strains belonged to type I, four strains to G and three strains to a novel RFLP type, designated AD. Phylogenetic analysis confirmed that type AD isolates clustered closely with *B. cepacia* genomovar III-B strains (Fig. 1). The *B. cepacia* genomovar III isolates did not react with primers specific for other genomovars.

All *B. multivorans* and *B. ambifaria* isolates examined generated an amplicon with their respective primer pair (100% sensitivity). No cross-reactions were observed with primer pairs specific for other genomovars.

None of the *B. stabilis*, *B. vietnamiensis*, *B. cepacia* genomovar VI and *B. anthina* isolates reacted with the primer pairs studied. However, all seven *B. pyrrocinia* isolates gave positive results in the *B. cepacia* genomovar I specific PCR test; no amplification products were generated in the other PCR assays.

---

**Fig. 1.** Phylogenetic tree of *recA* sequences from strains of the current *B. cepacia* complex species. The tree was constructed as described in Materials and methods. Genetic distance is shown on the scale and bootstrap analysis for node values >70% are indicated. The *B. cepacia* complex genomovar or species, and the positions of strains of novel *recA* RFLP type AD, AW and K are indicated.
The three isolates (with identical RFLP profiles) which were not assigned to one of the B. cepacia complex genomovars (B. ubonensis LMG 20358 and two B. cepacia-like strains) gave negative results in all of the PCR tests examined. The fact that these isolates generated a recA amplicon with the B. cepacia complex specific primers suggested that they are related to the complex. These findings confirmed the high level of 16S rDNA similarity reported between B. ubonensis and other B. cepacia complex bacteria [28]. Further taxonomic work is required to clarify the status of these isolates within the B. cepacia complex.

In summary, the recA-based PCR tests examined proved to be excellent diagnostic tools for the identification of B. cepacia complex members. They allowed identification of the whole B. cepacia complex as a group and of B. multivorans and B. cepacia genomovar III, the prominent organisms in CF specimens. However, the sensitivity and specificity of the B. cepacia genomovar I assay should be improved, particularly for environmental samples. The diverse strain collection examined in the present study revealed several new recA RFLP types within the B. cepacia complex and included one CF and one environmental isolate that were indistinguishable from B. ubonensis, another putative member of the B. cepacia complex.

We are indebted to the Fund for Scientific Research - Flanders (PV) and the UK Cystic Fibrosis Trust (E.M. and PV) for financial support. E.M. is grateful to Julie Fadden for excellent technical assistance.

References