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Possibilities in Identification of Genomic Species of *Burkholderia cepacia* Complex by PCR and RFLP

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Abstract

The strains belonging to *Burkholderia cepacia* complex are important opportunistic pathogens in immunocompromised patients and cause serious diseases. It is possible to obtain isolates from soil, water, plants and human samples. Taxonomy of this group is difficult. *Burkholderia cepacia* complex consists of seventeen genomic species and the genetic scheme is based on *recA* gene. Commonly, first five genomovars occurre in humans, mostly genomovars II and III, subdivision IIIA. Within this study we tested identification of first five genomovars by PCR with following melting analysis and RFLP. The experiments were targeted on eubacterial 16S rDNA and specific gene *recA*, which allowed identification of all five genomovars. *RecA* gene appeared as more suitable than 16S rDNA, which enabled direct identification of only genomovars II and V; genomovars I, III and IV were similar within 16S rDNA sequence.

Key words: *recA*, 16S rDNA, HRMA

Introduction

Mapping and dissemination of infection agents are an important topic in public health and epidemiology. Molecular methods have contributed to recent advances in the tracking of the nosocomial and environmental spread of pathogenic bacteria. These methods enable genetic identification of microbes at the level of subspecific strains (Belkum *et al*., 2002).

Burkholderia cepacia complex (Bcc) is a very diverse group of bacteria, whose members are opportunistic human pathogens that can cause infections in immunocompromised patient, for example with cystic fibrosis and in other people with immune deficiency (Govan *et al*., 1996). Species from the Bcc can be transmitted between patients and are frequently resistant to wide range antibiotics. In addition, infection with these strains can cause a "cepacia syndrome" or a necrotizing pneumonia with bacteremia which leads to an acute and frequently fatal clinical decline (Frangolias *et al*., 1999; Isles *et al*., 1984). Distinction of genomic species in the Bcc by routine clinical microbiology methods is difficult. There are available phenotypic tests to identify genomovar II (species *B. multivorans*), genomovar IV (species *B. stabilis*) and genomovar V (species *B. vietnamensis*) (Whitby *et al*., 2000). From epidemiological point of view, it is important to distinguish genomovar II (species *B. multivorans*) and III (species *B. cenocepacia*) as infections caused by genomovar II can evolve into fatal "cepacia syndrome". The two above mentioned genomovars are more virulent than other belonging to complex. Identification of genomovars can be useful for treatment purposes (Zahariadis *et al*., 2003; Nash *et al*., 2011).

Aim of our study was to verify an applicability of the previously published systems for identification of genomovars belonging to the Bcc (Whitby *et al*., 2000; Mahenthiralingam *et al*., 2000 and Dřevínek *et al*., 2002). These systems use agarose gel electrophoresis as post-PCR analysis, however, we verified possibility of using melting analysis, which is closed system, so there is no possible cross contamination, reduced sample handling and the method is less time-consuming.

Experimental

Materials and Methods

Bacterial isolates. 145 clinical isolates from collection of Department of Microbiology, Faculty of Medicine and Dentistry, Palacký University Olomouc and Faculty Hospital Olomouc and 5 reference strains from

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The Belgium Coordinated Collections of Microorganism/Laboratorium Microbiologie Ghent (BCCM/LMG) LMG 1222, LMG 13010, LMG 16656, LMG 14294, LMG 10929 were used in this study. Strains were cultivated overnight on blood agar for DNA extraction.

DNA extraction. Chromosomal DNA was extracted from all strains by using heat extraction. First, colony was resuspended in 50 µl of deionized water, incubated at 94°C for 10 min, centrifuged by 13 000 × g for 4 min and then 25 µl of supernatant was transferred into new tube. Extracted DNA was used directly as template for PCR or kept at –20°C until needed.

PCR analysis. Each 20 µl reaction contained 14.24 µl deionized water, 2 µl buffer complete (final concentration of $MgCl_2$ was 1.5 mM), 1 µl LCGreen Plus, 0.1 µl of primer (final concentration 0.5 µM, primer pair to PCR system choose according Table I), 0.16 µl dNTPs (25 mM stock, final concentration 0.2 mM), 0.4 *Taq*-polymerase (2 U/reaction) and 2 µl DNA template. For real-time PCR amplification with following High-resolution melting analysis (HRMA) was performed using Rotor-Gene 6000 (Qiagen). Thermal cycling parameters for PCR system A and C included a pre-denaturation at 94°C for 7 min followed by 40 cycles of 30 sec at 94°C (denaturation), 45 sec at 62°C (annealing) and 60 sec at 72°C (extension) and final extension 7 min at 72°C and melting in range from 55°C to 95°C. Fluorescence data were acquired at 0.03°C increments.

Rotor-Gene software (version 2.0.2.4) enabled visualisation of HRM data. The negative derivative of fluorescence over temperature (df/dt) curve displays melting temperature (Tm), the normalized curve represented the decreasing fluorescence versus increasing temperature (Wittwer *et al.*, 2003). For distinguish of strains we used derivative curves with melting temperature (Tm).

Thermal cycling parameters for PCR system B included a pre-denaturation at 94°C for 7 min followed by 30 cycles of 45 sec at 94°C (denaturation), 45 sec at 66°C (annealing) and 120 sec at 72°C (extension) and final extension 7 min at 72°C.

Results from PCR system B were evaluated on the basis of negativity or positivity, according algorithm to identify genomovars of the Bcc, published earlier in Whitby *et al.* (2000).

For restriction fragment length polymorphism (RFLP) analysis, 10 µl of PCR product amplified by PCR

Table I
Primers for PCR and RFLP used in experiments.

PCR system	Specificity	Primer name	Primer sequence (5' to 3')	Target	Reference
A	Genomovar I (*B. cepacia*)	BCRG11	CAGGTCGTCTCCACGGGT	*recA*	Mahenthiralingam *et al.*, 2000; Dřevínek *et al.*, 2002
		BCRG12	CACGCCGATCTTCATACGA		
	Genomovar II (*B. multivorans*)	BCRBM1	CGGCGTCAACGTGCCGGAT		
		BCRBM2	TCCATCGCCTCGGCTTCGT		
	Genomovar IIIA (*B. cenocepacia*)	BCRG3A1	GCTCGACGTTCAATATGCC		
		BCRG3A2	TCGAGACGCACCGACGAG		
	Genomovar IIIB (*B. cenocepacia*)	BCRG3B1	GCTGCAAGTCATCGCTGAA		
		BCRG3B2	TACGCCATCGGGCATGCT		
	Genomovar IV (*B. stabilis*)	BCRG41	ACCGGCGAGCAGGCGCTT		
		BCRG42	ACGCCATCGGGCATGGCA		
	Genomovar V (*B. vietnamensis*)	BCRBV1	GGGCGACGGCGACGTGAA		
		BCRBV2	TCGGCCTTCGGCACCAGT		
B	Genomovar I Genomovar III	G1	GCCATGGATACTCCAAAAGGA	23S rDNA	Whitby *et al.*, 2000
	Genomovar IV	G2	TCGGAATCCTGCTGAGAGGC	16S rDNA	
	Genomovar I Genomovar II Genomovar III	SPR3	TCGAAAGAGAACCGGCG		
	Genomovar I Genomovar IV	SPR4	TCGAAAGAGAACCGATA		
C	Universal	UNI2	GACTCCTACGGGAGGCAGCAG	16S rDNA	Mahenthiralingam *et al.*, 2000
		UNI5	CTGATCCGCGATTACTAGCGATTC		
	B. cepacia complex	BCR1	TGACCGCCGAGAAGAGCAA	*recA*	
		BCR2	CTCTTCTTCGTCCATCGCCTC		

system C was combined with deionized water (17 µl), the appropriate restriction enzyme buffer (2 µl) and endonuclease *HaeIII* (1 µl) and incubated at 37°C for 7 min. RFLP products were analysed by agarose gel electrophoresis, with agarose concentration 2%. Molecular size marker of the appropriate size range was included on all gels (100 bp DNA ladder or 50 bp DNA ladder).

Results

We tested the utility of PCR and RFLP to distinguish first five genomovars of Bcc. These methods were tested on 145 clinical isolates and five reference strains from BCCM/LMG. Only one clinical isolate belonged to genomovar I, 109 clinical isolates to genomovar II and 35 to genomovar III, subdivision IIIA.

Genomovar specific amplification (PCR system A), Bcc specific PCR, which was aimed on *recA* gene, provided higher resolution capability. This method enables to distinguish all five tested genomovars and within genomovar III it was possible differentiate two subdivisions IIIA and IIIB. The identification by the PCR system A was based on the presence of the specific peak (Fig. 1). Results from melting analysis were supported with results from agarose gel electrophoresis, for verify usability of melting analysis for determination positive or negative reaction.

The system published by Whitby *et al.* (2000) (PCR system B) targeted 16S/23S rDNA within two PCR with primer pairs G1-G2 and SPR3-G1 or SPR4-G1. Primer pairs G1-G2 enabled to distinguish genomovars I, III and IV from genomovars II and V. Second PCR with primer pair SPR3-G1 or SPR4-G1 allowed identification of genomovars I/III, I/IV, II and V. It was possible to distinguish genomovars II and V according the PCR system B.

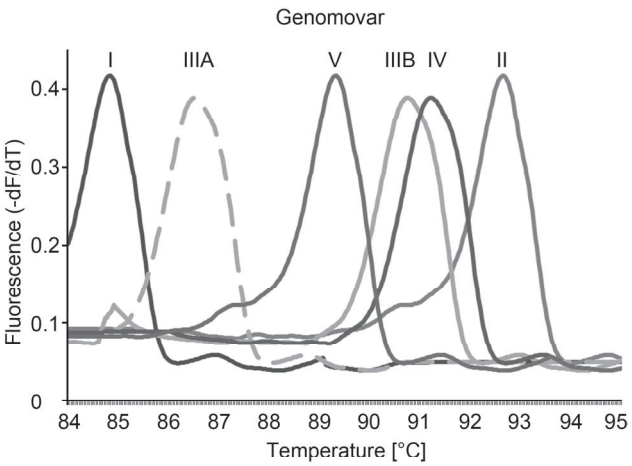

Fig. 1. Results of genomovar specific PCR-HRMA of five genomovars from *Burkholderia cepacia* complex.

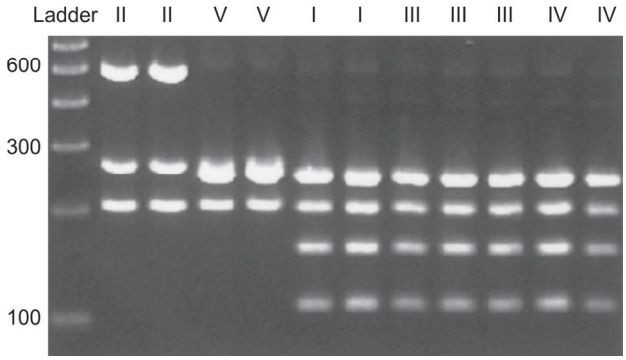

Fig. 2. RFLP analysis with *HaeIII* of 16S rDNA of five genomovars from *Burkholderia cepacia* complex.

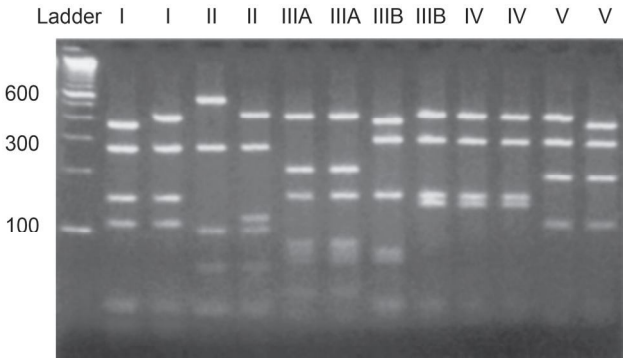

Fig. 3. RFLP analysis with *HaeIII* of *recA* gene of five genomovars from *Burkholderia cepacia* complex.

PCR system C with following RFLP analysis with *HaeIII* of PCR-amplicons of 16S rDNA revealed sequence polymorphism capable of identifying genomovars II and V but was insufficient to discriminate genomovars I, III and IV (Fig. 2). RFLP analysis with *HaeIII* of PCR-amplicons of *recA* gene was enough sufficient nucleotide sequence variation to distinction of all tested genomovars I, II, III, IV and V (Fig. 3). Isolates of genomovar III included two the subdivision IIIA and IIIB after cleavage *recA* by *HaeIII*.

At first we demonstrated that the melting analysis can replace agarose gel electrophoresis as post-PCR analysis. Analysis of *recA* gene by the PCR system A with following melting analysis or RFLP achieved the same discrimination power, and analysis of 16S rDNA by the PCR system B with following melting analysis or RFLP gave the same results. The differentiation of genomovar II (species *B. multivorans*) was possible by using the PCR system A, B or RFLP aiming16S rDNA and *recA* genes. Genomovar III (species *B. cenocepacia*) was directly identified only by genomovar specific PCR (PCR system A) or RFLP with *HaeIII* aimed at *recA* gene. Finally, we proved that the analysis of specific gene is more suitable for identification and distinction of Bcc than eubacterial gene, which we can find by all bacterial species.

Discussion

Several studies have indicated problems with right identification of Bcc by phenotypic methods. The molecular methods are more reliable in this field (Henry *et al.*, 1997; McMenamin *et al.*, 2000). Within the genetic identification we aim for molecular marker *recA* gene and 16S rDNA, which is very extensive for high degree of conservation and included variable regions (Liu *et al.*, 2012).

Our results of PCR with following melting analysis or RFLP analysis were same as in studies Whitby *et al.*, 2000; Mahenthiralingam *et al.*, 2000a; McDowell *et al.*, 2001 and Mahenthiralingam *et al.*, 2002. Studies Mahenthiralingam *et al.* (2000b) and Dřevínek *et al.* (2002) describing the development of genomovar specific PCR. We tested their conclusions in practice and we compared it with PCR and RFLP aimed at eubacterial 16S rDNA. Genomovar specific PCR and RFLP focused on *recA* gene had better discriminating power.

We had not enough isolates of genomovar IIIB and IV (they are not easy accessible) so we cannot surely conclude that according results from melting analysis that the distinction of curves appertain to genomovar IIIB and IV is significant. We recommend verifying the results of identification of genomovars by agarose gel electrophoresis, size of PCR product is different, for IIIB is 781 bp and for IV is 647 bp.

Whitby *et al.* (2000) developed PCR reactions focused on 16S rDNA and 23S rDNA for identification of Bcc. PCR was scored based on positive and negative reactions, described method enabled to distinguish genomovars II and V, which had differences in sequence of 16S rDNA and it was possible to suggest primers for dissimilar regions. Sequences of genomovar I was similar to genomovar II and IV.

We focused our experiments only on first five genomovars, which occur with the highest frequency. Their identification and discrimination would be beneficial as it includes two genetic types also that cause most complications in infected (occurrence of antibiotic resistance, cepacia syndrome). These are genomovar II and genomovar III and they are two the most commonly isolated genomovars too. These two genomovars are significant for epidemiology (Mahenthiralingam *et al.*, 2002 and Jones *et al.*, 2003).

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