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ORIGINAL PAPER

Evaluation of different molecular and phenotypic methods for identification of environmental *Burkholderia cepacia* complex

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Abstract

The correct identification of different genera and bacterial species is essential, especially when these bacteria cause infections and appropriate therapies need to be chosen. Bacteria belonging to the *Burkholderia cepacia* complex are considered important opportunistic pathogens, causing different types of infections in immunocompromised, principally in patients with cystic fibrosis. Twenty-one isolates were obtained from different soil samples and identified by sequencing of 16S rRNA, 23S rRNA, *rec*A gene, MLST and by VITEK 2 and MALDI-TOF MS systems. Then, statistical analyses were performed. VITEK 2 and MALDI-TOF MS systems showed different bacterial genera. Sequencing of the 16S rRNA, 23S rRNA gene and amplification of *rec*A gene showed that all the isolates belong to the *B. cepacia* complex. Sequencing of the *rec*A gene showed a predominance of *B. cenocepacia*. The PCR of the *rec*A gene showed a high specificity when it is necessary to identify the bacteria belonging to the *B. cepacia* complex in comparison with 16S and 23S rRNA genes sequencing. MLST analyzes showed a diversity of STs, which have not yet been correlated to the species. Phenotypic identification was not suitable for the identification of these pathogens since in many cases different genera have been reported, including identification by using MALDI-TOF MS.

Keywords *Burkholderia cepacia* complex · 16S rRNA · 23S rRNA · *rec*A · MLST · MALDI-TOF MS

Introduction

The *Burkholderia cepacia* complex is a group of 21 different species widely found in the environment (Sousa et al. 2010; Peeters et al. 2013; De Smet et al. 2015; Martina et al. 2018). Species of the *B. cepacia* complex are used in bioremediation and biotechnology; however, in the hospital environment they are considered important opportunistic pathogens, causing infections in immunocompromised patients, principally in patients with cystic fibrosis (CF) (Uehlinger et al. 2009). *B. cepacia* complex stands out due to different resistance mechanisms, which confer non-susceptibility to the most of available antibiotics. In patients with CF, these pathogens are associated with a high level of morbidity and mortality. Moreover, outbreaks of different species of the *B. cepacia* complex have been reported (Chiarini et al. 2006; Leitão et al. 2008; Sousa et al. 2010).

The bacterial identification is in increasing evolution and different phenotypic and genotypic methods and also automated and semi-automated systems are used to identify bacterial genera and species. Bacteria belonging to the *B. cepacia* complex have a high level of similarity among them, making it difficult the correct identification of these pathogens, since incorrect identification may lead to inappropriate choice of antibiotics for the therapeutic purpose. Besides that, the correct identification of bacteria belonging to *B. cepacia* complex, mainly *B. cenocepacia* is of great importance due to contraindication of these pathogens in lung transplantation in CF patients (Malini et al. 2009; Lipuma et al. 2010; Chawla et al. 2013).

The incorrect identification of *B. cepacia* complex as *Burkholderia pseudomallei* complex represents a public health risk since *B. pseudomallei* complex causes serious

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infections, such as melioidosis and glanders. Thus, the discrimination between these two complexes is of great importance, affecting the decision-making process referent to safety and therapeutic choice (Gilling et al. 2014). This study aimed to compare distinct methods used for bacterial identification, such as sequencing of 16S rRNA, 23S rRNA and *rec*A genes, the automated systems Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS), Multilocus sequence typing (MLST) and VITEK 2 (bioMérieux, France) in bacteria belonging to the *B. cepacia* complex isolated from soil samples.

Materials and methods

Obtaining of isolates

Several soil samples from different cities, states and cultures were obtained for the bacterial isolation The bacterial isolates were obtained according to Mukherjee et al. (2011), using MacConkey Agar (Oxoid, United Kingdom) and they were stocked at − 80 °C in the Brain Heart Infusion (BHI) broth (Oxoid, United Kingdom) with glycerol 15%.

Control strains

Burkholderia cepacia ATCC® 25416, *B. cepacia* ATCC® 17759, *Pseudomonas aeruginosa* ATCC® 27853, *Klebsiella pneumoniae* ATCC® BAA 1705 and *Escherichia coli* ATCC® 25922 strains were used as control in all experiments.

VITEK® 2 system

The isolates were identified using VITEK® 2 automated instrument ID system (bioMérieux, USA) with the GN card, which has 64 biochemical tests and identifies a total of 135 taxa, 68 being non-*Enterobacteriaceae*. Colonies of a pure culture were seeded on MacConkey Agar plates (Oxoid, United Kingdom) and incubated at 37 °C for 18–24 h. Subsequently, a bacterial suspension was performed, which was adjusted according to the McFarland scale (0.5–0.63) in 0.9% sodium chloride solution using the VITEK 2 DensiCheck instrument (bioMérieux, USA). The cassettes containing the GN cards were carried to the VITEK® 2 instrument and the cards were read by the system.

MALDI-TOF MS system

The isolates were identified using the MALDI-TOF VITEK® MS (bioMérieux, USA). Colonies of a pure culture were seeded on MacConkey Agar plates (Oxoid, United Kingdom) and incubated at 37 °C for 18–24 h. Subsequently, bacterial cells were added in the spots on the target slide using the inoculation loop method and then, 1 µL of the VITEK MS-CHCA matrix was added. The target slide was added to the VITEK MS and the mass spectra of whole bacterial cell protein were generated and analyzed.

Genomic DNA extraction

The genomic DNA was extracted using the QIAamp DNA Mini Kit (QIAGEN, Germany). Concentration and purity were determined by DS-11 + Spectrophotometer (DeNovix, USA).

Sequencing of 16S rRNA

PCR reactions were performed for amplification of the 16S rRNA using the primers fd1 (5′-AGAGTTTGATCCTGG CTCAG-3′) and fd2 (5′-ACGGCTACCTTGTTACGACTT-3′) according to Weisburg et al. (1991). All PCR reactions were performed using 100 ng of genomic DNA and 1.25U of JumpStart™ Taq DNA Polymerase (Sigma-Aldrich, USA) on a ProFlex™ PCR Thermocycler System (Applied Biosystems, Singapore). The amplicons were sequenced using BigDye Terminator Cycle Sequencing Kit on an ABI 3500xL Genetic Analyzer (Applied Biosystems, USA).

Sequencing of 23S rRNA

PCR reactions were performed for amplification of the 23S rRNA using the primers 23SrRNA-For (5′-CYGAAT GGGGVAACC-3′) and 23SrRNA-Rev (5′-CGACATCGA GGTGCCAAAC-3′) according to Hunt et al. (2006). All PCR reactions were performed using 100 ng of genomic DNA and 1.25U of JumpStart™ Taq DNA Polymerase (Sigma-Aldrich, USA) in ProFlex™ PCR Thermocycler System (Applied Biosystems, Singapore).

Amplification and sequencing of *rec*A gene

PCR reactions were performed for amplification of the *rec*A gene using specific primers BUR1 (5′- GATCGARAAGCA GTTCGGCAA-3′) and BUR2 (5′- TTGTCCTTGCCCTGR CCGAT-3′) for *Burkholderia* genus, according to Payne et al. (2005). The specific primers BCR1 (5′-TGACCGCCG AGAAGAGCAA-3′) and BCR2 (5′-CTCTTCTTCGTCCAT CGCCTC-3′) were used for *B. cepacia* complex according to Mahenthiralingam et al. (2000). All PCR reactions were performed using 100 ng of genomic DNA and 1.25U of JumpStart™ Taq DNA Polymerase (Sigma-Aldrich, USA) on a ProFlex™ PCR Thermocycler System (Applied Biosystems, Singapore). Sequencing of the *rec*A gene was performed using combinations of primers BCR1 (5′- TGACCGCCG AGAAGAGCAA-3′) with BCR4 (5′- GCGCAGCGCCTG

CGACAT-3′) and BCR2 (5′- CTCTTCTTCGTCCATCGC CTC-3′) with BCR3 (5′- GTCGCAGGCGCTGCGCAA-3′), according to Mahenthiralingam et al. (2000).

Multilocus sequence typing (MLST)

Amplification and sequencing of *atp*D, *glt*B, *gyr*B, *rec*A, *lep*A, *pha*C and *trp*B genes were performed using the updated primers and conditions according to Spilker et al. (2009). MLST analyzes were performed using the *B. cepacia* complex MLST Databases sited at the University of Oxford (https://pubmlst.org/bcc/). All PCR reactions were performed using 100 ng of genomic DNA and 1.25U of JumpStart™ Taq DNA Polymerase (Sigma-Aldrich, USA) on a ProFlex™ PCR Thermocycler System (Applied Biosystems, Singapore).

Sequences analyses

The sequences analyses were performed using ChromasPro version 1.7.6 software (Technelysium Pty. Ltd) and then, they were compared to sequences available in GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Clustal Omega EMBL-EMI Multiple Sequence Alignment (http://www.ebi.ac.uk/Tools/msa/clustalo/) was used to the sequences alignments.

Statistical analyses

Statistical analyzes were performed according to Landis and Koch (1977) using the kappa (k) test. The criteria of agreement were established as almost perfect (k: 0.81–1.00), substantial (k: 0.61–0.80), moderate (k: 0.41–0.60), reasonable (k: 0.21–0.40) and slight (k: 0.00–0.20).

Results

Identification of isolates using VITEK 2 system

The identification by VITEK 2 showed a diversity of genera and species, being eight isolates identified as *B. cepacia*, five as *B. cepacia* group, three as *B. pseudomallei* and *Ochrobactrum anthropi* and the S674 isolate was identified as *Rhizobium radiobacter*. Two isolates were unidentified (Table 1).

Identification of isolates using 16S and 23S rRNA sequencing

Sequencing of the 16S rRNA and 23S rRNA genes showed that all the isolates belong to the *Burkholderia* genus, so all were identified as *B. cepacia* complex (Table 1).

Identification of isolates using amplification and sequencing of *rec*A gene

Amplification of *rec*A gene showed that all isolates belong to the *Burkholderia* genus and *B. cepacia* complex. Sequencing of the *rec*A gene showed a predominance of *B. cenocepacia*, since 15 (68.1%) of the isolates were identified as this species. Among the other seven isolates, three were identified as *B. cepacia* and two as *B. lata* and *B. ambifaria*.

Identification of isolates using MALDI-TOF MS system

MALDI-TOF MS results showed a variety of three different bacteria, such as *B. cepacia* (12), *O. anthropi* (9) and *S. maltophilia* (1) (Table 1).

Identification of isolates using MLST

MLST analyzes showed a diversity of STs, being ST1318 (8) the most prevalent, followed by ST533 (4), ST1440 (2), ST787 (2), ST743 (1), ST789 (1), ST1034 (1), ST1345 (1), ST295 (1) and ST662 (1) (Table 1).

Statistical analysis

Statistical analysis using *rec*A sequencing for identification of *Burkholderia* species showed slight agreement with VITEK 2 (k: 0.002) and MALDI-TOF MS (k: 0.018). Results of *rec*A gene PCR for identification of *B. cepacia* complex showed almost perfect agreement with 16S rRNA and 23S rRNA gene sequencing. MLST analysis for identification of *B. cepacia* complex showed reasonable agreement with VITEK 2 (k: 0.34) and MALDI-TOF MS (k: 0.29) (Table 2).

Discussion

The correct identification of different genera and bacterial species is essential, especially when these bacteria cause infections and appropriate therapies need to be chosen. Different studies show the comparison of different methods used for bacterial identification, however, the great majority is carried out with clinical bacteria (Guo et al. 2014; Febbraro et al. 2016).

Among non-fermenting Gram-negative bacilli (NFGNB), which are widely distributed in the environment, the *B. cepacia* complex stands out due to the high phenotypic and genotypic similarity among the 21 species described (Sousa et al. 2010; Peeters et al. 2013; De Smet et al. 2015; Abbott and Peleg 2015; Martina et al. 2018). The species of the *B. cepacia* complex have been reported

Table 1 Identification of *B. cepacia* complex isolated from soil using different methods

Isolate	VITEK 2	16S rRNA gene sequencing	23S rRNA gene sequencing	*recA* PCR		*recA* gene sequencing	MLST (ST)	MALDI-TOF MS
				Bg	Bcc			
S673	*B. cepacia* group	*B. cepacia* complex	*B. cepacia* complex	+	+	*B. lata*	1440	*Ochrobactrum anthropi*
S674	*Rhizobium radiobacter*	*B. cepacia* complex	*B. cepacia* complex	+	+	*B. cenocepacia*	1318	*Stenotrophomonas maltophilia*
S675	*B. cepacia* group	*B. cepacia* complex	*B. cepacia* complex	+	+	*B. cenocepacia*	1318	*B. cepacia*
S677	*B. pseudomallei*	*B. cepacia* complex	*B. cepacia* complex	+	+	*B. cenocepacia*	1318	*B. cepacia*
S678	*B. pseudomallei*	*B. cepacia* complex	*B. cepacia* complex	+	+	*B. cenocepacia*	1318	*Ochrobactrum anthropi*
S679	Unidentified	*B. cepacia* complex	*B. cepacia* complex	+	+	*B. cenocepacia*	1034	*Ochrobactrum anthropi*
S680	*Ochrobactrum anthropi*	*B. cepacia* complex	*B. cepacia* complex	+	+	*B. cenocepacia*	1318	*Ochrobactrum anthropi*
S682	*B. cepacia*	*B. cepacia* complex	*B. cepacia* complex	+	+	*B. cenocepacia*	1345	*B. cepacia*
S683	*Ochrobactrum anthropi*	*B. cepacia* complex	*B. cepacia* complex	+	+	*B. cenocepacia*	662	*Ochrobactrum anthropi*
S684	*B. cepacia* group	*B. cepacia* complex	*B. cepacia* complex	+	+	*B. cenocepacia*	1318	*Ochrobactrum anthropi*
S685	*B. cepacia*	*B. cepacia* complex	*B. cepacia* complex	+	+	*B. lata*	1440	*Ochrobactrum anthropi*
S686	*B. cepacia*	*B. cepacia* complex	*B. cepacia* complex	+	+	*B. cepacia*	787	*B. cepacia*
S687	*B. cepacia* group	*B. cepacia* complex	*B. cepacia* complex	+	+	*B. cepacia*	789	*B. cepacia*
S688	*B. cepacia* group	*B. cepacia* complex	*B. cepacia* complex	+	+	*B. cenocepacia*	533	*B. cepacia*
S689	*B. pseudomallei*	*B. cepacia* complex	*B. cepacia* complex	+	+	*B. cepacia*	787	*B. cepacia*
S690	Unidentified	*B. cepacia* complex	*B. cepacia* complex	+	+	*B. cenocepacia*	533	*Ochrobactrum anthropi*
S691	*B. cepacia*	*B. cepacia* complex	*B. cepacia* complex	+	+	*B. ambifaria*	295	*B. cepacia*
S692	*Ochrobactrum anthropi*	*B. cepacia* complex	*B. cepacia* complex	+	+	*B. cenocepacia*	1318	*B. cepacia*
S693	*B. cepacia*	*B. cepacia* complex	*B. cepacia* complex	+	+	*B. ambifaria*	743	*B. cepacia*
S694	*B. cepacia*	*B. cepacia* complex	*B. cepacia* complex	+	+	*B. cenocepacia*	533	*B. cepacia*
S695	*B. cepacia*	*B. cepacia* complex	*B. cepacia* complex	+	+	*B. cenocepacia*	1318	*Ochrobactrum anthropi*
S696	*B. cepacia*	*B. cepacia* complex	*B. cepacia* complex	+	+	*B. cenocepacia*	533	*B. cepacia*

Bg Burkholderia genus; *Bcc B. cepacia* complex; *MLST* multilocus sequence typing; *MALDI-TOF* matrix-assisted laser desorption/ionization time-of-flight

Table 2 Evaluation of agreement of the different methods used

Tests	k	Agreement[a]
23S rRNA sequencing vs. *recA* PCR	1.00	Almost perfect
16S rRNA sequencing vs. *recA* PCR	1.00	Almost perfect
VITEK 2 vs. MLST	0.34	Reasonable
MALDI-TOF vs. MLST	0.29	Reasonable
VITEK 2 vs. *recA* sequencing	0.002	Slight
MALDI-TOF vs. *recA* sequencing	0.018	Slight

k kappa

[a]agreement according to (Landis and Koch 1977)

in different sources, such as humans (CF and non-CF), rhizosphere soil, plant, sheep, river water, hospital and industrial contaminants (Vandamme and Dawyndt 2011).

Sequencing of *rec*A gene was determined as an efficient method used to identify species belonging to the *B. cepacia* complex (Payne et al. 2005); however, not all laboratories have molecular biology facilities. Most laboratories use combinations of phenotypic methods for bacterial identification, including the most recent method of MALDI-TOF MS, which is a rapid and practical method. The sequencing of 16S and 23S rRNA genes are widely used for bacterial identification, however, for bacteria belonging to the *B. cepacia* complex, these methods do

not achieve species differentiation efficiently (Eisen 1995; Karlin 1995).

The comparative analyses among the phenotypic methods used in the present study with the *rec*A gene for *Burkholderia* species indicated that both methods erroneously identified several isolates. VITEK 2 system did not identified two isolates (S679 and S690) and showed also different bacterial genera to some isolates, whereas MALDI-TOF MS erroneously identified 11 isolates as different bacterial genera. Several studies have shown a low specificity for different bacterial genera in automated phenotypic methods when compared with molecular methods, however, MALDI-TOF MS has been shown to be very useful (Bosshard et al. 2006; Guo et al. 2014; He et al. 2016; Febbraro et al. 2016).

Unlike Fehlberg et al. (2013) and Vicenzi et al. (2016) who reported that in clinical isolates of the *B. cepacia* complex the identification by these phenotypic methods was adequate, in the environmental isolates of the present study, these methods showed a low specificity and cannot be indicated for identification of this bacterial complex. Studies conducted by Vandamme and Dawynd (2011) and Navrátilová et al. (2013) showed a difficulty in differentiating some *Burkholderia* species, such as *B. cepacia* from *B. cenocepacia*, which may have happened in this study, since most of the isolates were identified as *B. cepacia* by MALDI-TOF MS and *B. cenocepacia* by sequencing of *rec*A gene.

The comparative analyses among the genotypic methods for *B. cepacia* complex indicated that the sequencing of 16S and 23S rRNA genes showed a similarity ≥ 97% with different species belonging to the *B. cepacia* complex since all isolates were characterized by *rec*A PCR as *B. cepacia* complex. Studies have shown that by sequencing of these genes it is possible to differentiate the *Burkholderia* genus from other genera; however, it is not possible to differentiate the species (McDowell et al. 2001; Payne et al. 2005).

MLST analyzes revealed that only three STs found were characterized at species level, being one of them for *B. cenocepacia* (ST662) and two other for *B. ambifaria* (ST295) and ST743). Thus, it was not possible to correlate the MLST results at the species level with those obtained in the other methods. MLST is used worldwide since it is often possible to discriminate the species belonging to this complex; however, because of the great genetic diversity among these species many STs are not yet characterized and other molecular methods need to be performed, although it can provide a better understanding of diversity and population dynamics (Spilker et al. 2009; Vandamme and Dawyndt 2011).

There are no studies comparing identification of *B. cepacia* complex isolated from soil by different methods. The bacteria belonging to the *B. cepacia* complex are opportunistic and distributed in different environments and erroneous identification can cause serious problems, especially when these environmental pathogens enter the hospital environment and cause infections in immunocompromised patients.

In conclusion, the PCR of the *rec*A gene showed a high specificity when it is necessary to identify the bacteria belonging to the *B. cepacia* complex in comparison with 16S and 23S rRNA genes sequencing. Phenotypic identifications were not suitable for the identification of these pathogens since in many cases different genera have been reported. Further studies involving whole genome sequencing should be performed in order to characterize several STs at the species level. Therefore, it is important to expand the database of MALDI-TOF instruments by adding mass spectra of different type strains and several strains per species, thus avoiding errors in identification.

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