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Evaluation of MALDI-TOF MS System for the Identification and Differentiation *of Burkholderia cepacia* Complex Species

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HIGHLIGHTS

- MALDI-TOF is a feasible technique and presents a low cost of reagents.
- It can be used for the reliable differentiation of *B. cenocepacia* from other Bcc.
- Molecular techniques require a highly qualified workforce and demand time.
- MALDI-TOF identified all specimens at genus level and 96.2% to species level.

Abstract: To evaluate the performance of MALDI-TOF MS system as a tool for identification and differentiation of *Burkholderia cenocepacia* from other species of *Burkholderia cepacia complex* (Bcc). Fifty-three suggestive colonies were submitted to the MALDI-TOF Microflex LT 4.0® (Bruker Daltonik, Bremen, Germany) system for identification. We compared two protocols of protein extraction: (A) Direct Method and (B) Tube Extraction. In parallel, all isolates were subjected to molecular diagnosis (primers for recA gene) to identify species belonging to Bcc and to differentiate *B. cenocepacia* in genomovar IIIA or IIIB. MALDI-TOF was able to identify all isolates to the genus level and 94.3% (50/53) to species using both methods. The MALDI-TOF system was able to identify 38 out of the 40 isolates identified as *B. cenocepacia* by molecular

techniques. In conclusion, MALDI-TOF Microflex LT 4.0® is a feasible technique and presents a low cost of reagents; it can be used for the reliable differentiation of *B. cenocepacia* from other species of Bcc.

Keywords: *Burkholderia cepacia* complex; *Burkholderia cenocepacia*; MALDI-TOF MS; Polymerase Chain Reaction.

INTRODUCTION

Cystic fibrosis (CF) is an autosomal recessive genetic disease that affects several organs, and is mainly associated with chronic airway infection which can lead to intermittent pulmonary exacerbations. It is estimated that 80-95% of CF patients will develop respiratory failure due to chronic bacterial infections [1]. The microorganisms commonly associated with this pathology in the respiratory system are *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Haemophilus influenzae*, *Burkholderia cepacia* complex (Bcc) species, *Stenotrophomonas maltophilia*, and *Achromobacter xylosoxidans*, among other pathogens. Despite advances in the treatment of CF, infections due to bacteria belonging to Bcc still play an important role in the morbidity and mortality of these patients [2]. Airway infections due to Bcc are usually chronic, refractory to therapy due to resistance rates of Bcc isolates and related to a poor prognosis [3,4].

In 1992, a bacterium previously called *Pseudomonas cepacia* was reclassified as *Burkholderia cepacia* and a new genus was established [5]. Afterwards, with the improvement of molecular techniques, more species were included in this group of non-fermenters. Therefore, bacteria biochemically identified as *B. cepacia* consisted of at least five different genetically distinct species, named genomovars [5, 6]. Several species of this genus were grouped and identified as members of the Bcc, a very heterogeneous group of Gram negatives rods which is composed of 23 species. However, new members are often included in the complex [6, 7, 8].

Within the Bcc, the species *Burkholderia cenocepacia* stands out, due to its intraspecific diversity which leads to distinct genomovars: IIIA; IIIB; IIIC; IIID [2]. The interest in differentiating *B. cenocepacia* genomovars is associated with the clinical status of infected CF patients, as it can cause a necrotizing pulmonary infection, known as “cepacia syndrome”, with a high mortality rate [9]. Moreover, some strains of Bcc may present a high degree of transmissibility among CF patients [10, 11]. Thus, a few reference centers for the treatment of CF patients have established a physical barrier to preventing contagion among CF patients who are not colonized. Hence, the laboratories that attend CF centers must be able to identify the Bcc isolates using reliable techniques.

The differentiation of species of the Bcc cannot be achieved using traditional phenotypic methods in clinical microbiology laboratories. The main most sensitive discriminatory methodologies include molecular techniques such as the Polymerase Chain Reaction (PCR) with primers for the *rec-A* gene locus [12] or sequencing of the 16S rDNA. Although these methods are more accurate than phenotypic identification, they are expensive and require specialized professionals as well as proper equipment [13].

A technology that allows the culture-dependent identification of microorganisms with its speed, accuracy, practicality and low-cost of reagents is MALDI-TOF MS (Matrix-Assisted Laser Desorption-Ionization Time of Flight - Mass Spectrometry). This technology is based on evaluation of the protein profile (generated by the ionization of molecules) of a bacterium which is compared with a database of a standard proteins of bacterial profiles. MALDI-TOF is a very robust technique which allows the identification of bacteria, such as non-fermenters, with high accuracy; however, the ability to identify Bcc species using MALDI-TOF is variable. In fact, MALDI-TOF MS may not be able to identify intraspecific differences, such as *Burkholderia cenocepacia* and its genomovars [6]. In order to reduce the time for identification of *Burkholderia* species, the objective of this study was to evaluate the performance of the MALDI-TOF Microflex LT 4.0® system as a tool for the identification and differentiation of *Burkholderia cenocepacia* from other species of the *Burkholderia cepacia* complex.

MATERIAL AND METHODS

Bacterial specimens

A total of 53 colonies suggestive of Bcc obtained from routine sputum cultures of CF patients attending “Hospital de Clínicas de Porto Alegre (HCPA)” in Southern Brazil were collected from July 2020 to March 2021. All colonies were obtained from the *Burkholderia cepacia* Selective Agar (BCSA - Remel®, KS, USA) incubated for 24h-72h at 32ºC ± 2ºC. MALDI-TOF and PCR techniques were performed at “Laboratório de Pesquisa em Resistência Bacteriana (LABRESIS)” of HCPA. This cross-sectional and prospective study was

approved by the Comitê de Ética em Pesquisa of Hospital de Clínicas de Porto Alegre (CAAE 23417419.7.0000.5327).

MALDI-TOF MS

Prior to the identification in MALDI-TOF, two different methods of protein extraction were performed in duplicate with colonies grown on BCSA agar: (A) Direct Method and (B) Tube Extraction method. In method (A), 1µL of 70% formic acid was added later to the fixation of colonies in each target. After evaporation of the formic acid, 1µL of HCCA (α-cyano-4-hydroxycinnamic acid, Bruker Daltonik, Bremen, Germany) was pipetted and the sample was submitted to identification in MALDI-TOF Microflex LT 4.0® (Bruker Daltonik, Bremen, Germany). In method (B), the bacterial mass was transferred to a microtube to which 900µL of 100% ethanol was added. Afterwards, this microtube was centrifuged and the bacterial pellet was added to 25 µL of 70% formic acid and 25 µL acetonitrile. A volume of 1 µL of the extraction supernatant was placed on the target plate and, after evaporation, 1 µL of HCCA was added before it was submitted to MALDI-TOF using the same equipment as above. Before the identification of each isolate batch, the calibration process was performed with 1 µL of IVD Bacteria Test Standard (Bruker Daltonik, Bremen, Germany) and 1 µL of HCCA, followed by MALDI-TOF. Identification to species and genus levels were considered satisfactory for score of ≥2.0 and ≤1.99 to 1.70, respectively. All unsatisfactory results, which is defined as a score ≤1.69, were re-analyzed.

Extraction of DNA

All 53 colonies were submitted to a molecular technique (PCR) which was considered the reference method for the identification of Bcc species. The DNA of bacterial colonies was extracted by thermal lysis as follows: two or three colonies were suspended in 600µL of TE buffer (10 mM Tris-HCl (pH 8.0); 0.1 mM EDTA) and subjected to heat for 10 min at 100°C followed by cooling to -20°C for 20 min. The aliquots were centrifuged (Hermle Z 216MK, Hermle Labortechnik GmbH, Wehingen, Germany) for three minutes at 14,000 g and the supernatant (DNA) was stored at -20ºC in a microtube.

Polymerase Chain Reaction (PCR)

The PCR techniques were performed in duplicate. Initially, a PCR with BCR1 and BCR2 primers was used to generate amplicons which confirmed that the isolates were members of the *Burkholderia* genus. In order to confirm that the isolates belonged to the Bcc, a second PCR using the primers REC-IN5 (5'CATGATCGTCATCGACTCGGTC) and BCRBM2 (5'TCCATCGCCTCGGCTTCGT) was performed using the amplicons of the BCR1 (5'TGACCGCCGAGAAGAGCAA) and BCR2 (5'CTCTTCTTCGTCCATCGCCTC) [12]. Finally, a third PCR, also using the BCR1 and BCR2 amplicons, was performed with the primers BCRG3A1 (5'GCTCGACGTTCAATATGCC) and BCRG3A2 (5'TCGAGACGCACCGACGAG) for genomovar IIIA and BCRG3B1 (5'GCTGCAAGTCATCGCTGAA) and BCRG3B2 (5'TACGCCATCGGGCATGCT) for genomovar IIIB [12].

For the first PCR, the mix was composed of 5µL of 10x buffer, 2.5µL of MgCl2 and 5µL of dNTPs at a concentration of 2.5 mM. A volume of 20 picomoles of each primer (BCR1 and BCR2) was added in a final volume of 1.4µL with 25.7µL of water for PCR and 0.4µL of Taq Platinum DNA polymerase. The amplification program used an initial denaturation for 5 min at 95°C, 35 cycles of 45 seconds at 95°C, 45 seconds at 58°C and 90 seconds at 72°C, and a final extension of 10 minutes at 72°C at the end of the cycles.

The amplicons from the first PCR reaction were subjected to two other PCR reactions, as mentioned above. The second PCR reaction (for identification of species of the Bcc) used 5.0µL of 10x buffer solution, 1.5µL of 50 mM MgCl2 and 4.0µL of dNTP mixture at a concentration of 2.5mM. The primers REC-IN5 and BCRBM2 were added at a concentration of 10µM with 37.3µL of water for PCR, 0.2µL of Platinum Taq DNA polymerase and 2.0µL of the product of the first reaction. The program on the thermal cycler comprised theses stages: Stage 1 was 5 cycles of 30 seconds at 94°C, 45 seconds at 67°C, and 1 minute at 72°C; Stage 2 involved 5 cycles of 30 seconds at 94°C, 45 seconds at 65°C, and 1 minute at 72°C; Stage 3 was 15 cycles of 30 seconds at 94°C, 45 seconds at 63°C, and 1 minute at 72°C; all had a final extension of 5 minutes at 72°C.

The third PCR reaction (for *Burkholderia cenocepacia* genomovars IIIA and IIIB) used 2.5µL of the 10x buffer solution, 0.75µL of the 50mM MgCl2 and 2.0µL of the dNTP mixture. A volume of 1.0µL of each set of BCRG3A1 and BCRG3A2 primers was added at a concentration of 10uM, with 14.65µL of water for PCR, 0.1µL of Platinum Taq DNA polymerase and 3.0µL of the product of the first reaction. The thermocycler program was the same as in the second reaction.

The detection of the PCR reaction products was performed by visual inspection in an electrophoresis agarose gel. The amplicon of the second reaction (primers REC-IN5 and BCRBM2) was a 620bp DNA fragment which confirmed that the species belonged to the *Burkholderia cepacia* complex. The amplicon of the third reaction (primers BCRG3A1 and BCRG3A2) was a DNA fragment of 380bp corresponding to *Burkholderia cenocepacia* genomovar IIIA and a DNA fragment of 780bp corresponding to *B. cenocepacia* genomovar IIIB.

RESULTS

Identification by MALDI-TOF MS: comparison of the two extraction protocols

A total of 53 colonies suggestive of Bcc in the BCSA medium were submitted to identification by the MALDI-TOF system.

Regarding the protocols of protein exposure, both extractions, (A) and (B), presented excellent results to distinguish the isolates either to the genus level or to species level, as the two methods identified 100% (53/53) to genus level and 96.2% (51/53) to species level. In fact, 98.1% (52/53) of the results achieved an identification score using MALDI-TOF greater than 2.0 in at least one of the protocols of extraction. Only one isolate presented a score <2.0 for both protocols (score of 1.94 using protocol A and 1.72 using protocol B); this isolate, according to MALDI-TOF, was identified only to the "*Burkholderia* genus level" (Table 1).

When considering the efficiency of identification according to scores of MALDI-TOF, the tube extraction (protocol B) was shown to present better results than the direct method of extraction (protocol A). In fact, the extraction protocol B presented 50.9% (27/53) of scores ≥2.3, while extraction protocol A presented only 30.2% (16/53) of scores ≥2.3 (Table 1). The average score of identification was 2.23 for the direct method (A) and 2.27 for the tube extraction (B). In addition, one isolate (1.9% - 1/53) was not discriminated at the species level by the direct extraction and presented divergent results using the tube extraction. This isolate was sent for Sanger sequencing in order to confirm the species identification (result below).

Comparison of the MALDI-TOF identification with the molecular reference method (PCR)

The PCR techniques were able to classify 98.1% (52/53) of the isolates to the Bcc complex, with *B. cenocepacia* being the most common species (75.5%; 40/53). Only one isolate (1.9%; 1/53) was negative for Bcc according to molecular techniques (Table 2). One of the species identified by MALDI-TOF and which does not belong to Bcc was *B. gladioli*, which did not show amplification by the molecular method (Table 2).

One isolate classified as a member of the Bcc by PCR had its amplicon submitted to Sanger sequencing in order to confirm the species identification. This isolate presented an unsatisfactory identification by MALDI-TOF to the species level as follows: according to extraction protocol A, it was "*B. pyrrocinia*" (score 1.99), while according to protocol B, it was a "Member of the *Burkholderia cepacia* complex" (score 2.03). The results obtained by sequencing were compared with the GenBank database using the "National Center for Biotechnology Information Computer Blast" program (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The closer match observed with 100% identity in an overlap of 604 nucleotides occurred with the registration under the name "*B. contaminans* strain UFLA02-28 *RecA* (*recA*) gene, partial cds". Thus, the isolate was considered to be the species *B. contaminans* (isolated member of the Bcc).

The PCR for the differentiation of *B. cenocepacia* genomovars indicated that 56.6% (30/53) of the isolates corresponded to the IIIB genomovar and 18.9% (10/53) to the IIIA genomovar.

One isolate previously identified as *B. cepacia* (score according to protocols A and B were 2.36 and 2.30, respectively) in the MALDI-TOF system was identified as *B. cenocepacia* IIIB by the PCR. In addition, all species previously classified as belonging to Bcc and which were not *B. cenocepacia* did not present any amplification product in the specific PCR for genomovar differentiation.

Table 1. Comparison of protein extraction protocols using the numerical score for identification of species of *Burkholderia* genus by MALDI-TOF (classification at the species level and genus level was considered for score of ≥ 2.0 and ≥ 1.7≤1.99, respectively).

	***Score* of Direct Method (Protocol A)**			***Score* of Tube Extraction (Protocol B)**		
Species (n)	**1.94-1.99**	**2.0–2.29**	**2.3–2.38**	**1.74–1.99**	**2.0–2.29**	**2.3–2.44**
B. cenocepacia (39)	2	22	15	1	13	25
B. cepacia (6)	0	4	2	1	3	2
B. vietnamiensis (5)	0	4	1	0	2	3
B. gladioli (1)	0	0	1	0	0	1
B. multivorans (1)	0	1	0	0	0	1
B. lata (1)	0	1	0	0	1	0
Unsatisfactory identification (1)*	1	0	0	0	1	0

* This isolate presented an unsatisfactory identification by MALDI-TOF to specie level as follows: according to extraction protocol A as "*B. pyrrocinia*" (score 1.99) and according to protocol B as "Member of the *Burkholderia cepacia* Complex" (score 2.03).

Table 2. Comparison of identification by MALDI-TOF and the molecular technique (PCR) – (classification at the species level was considered for a score ≥ 2.0).

Identification by PCR (n)	**Identification by MALDI-TOF MS* (n)**	**Agreement**	**Sanger Sequencing**
Positive for Bcc			
B. cenocepacia IIIA (10)	*B. cenocepacia* (39)	97.5% (39/40)	-
B. cenocepacia IIIB (30)			-
Non- *B. cenocepacia* (12)	*B. cepacia* (4)	100% (4/4)	-
	B. vietnamiensis (5)	100% (5/5)	-
	B. multivorans (1)	100% (1/1)	-
	B. lata (1)	100% (1/1)	-
	Unsatisfactory identification (1) *	100% (1/1)	*B. contaminans*
Negative for Bcc			
	B. gladioli (1)	ND*	-

ND = Not determined; * This isolate presented an unsatisfactory identification by MALDI-TOF to specie level as folows: according to extraction protocol A as "*B. pyrrocinia*" (*score* 1.99) and according to protocol B as "Member of the *Burkholderia cepacia* Complex" (*score* 2.03).

DISCUSSION

This study aimed to evaluate the performance of the MALDI-TOF system as a toll for the identification and differentiation of *Burkholderia cenocepacia* species from the other Bcc members. We also compared different protein extraction protocols to be used prior to MALDI-TOF analysis. The average score of MALDI-TOF identification using the direct method (protocol A) and tube extractions (protocol B) was 2.23 and 2.27, respectively, which indicated that both extraction methods presented an excellent performance to distinguish the *Burkholderia* isolates to species level.

Although the identification based on extraction B presented higher scores when compared to extraction A, despite of that the latter protocol can properly be used as an extraction method with security of identification and without loss of quality [14]. In fact, the direct protocol (using only acid formic direct in plate) presents faster results and reduced costs when compared to the tube extraction technique [15, 14, 16]. Other authors have also reported the efficiency of the direct extraction for other non-fermenting bacteria [13, 17, 18, 19, 20, 21]. We would suggest using the direct method (A) in the routine of MALDI-TOF identification and the tube extraction only when there results of the direct methods present low scores as the tube extraction protocol allows the greater exposure of proteins and a better yield of identification.

The cut-off point for the precise species identification by MALDI-TOF is a widely discussed topic. Some authors suggest that a score ≥2.3 is more reliable for species identification [22, 23, 24]. In this study, 50.9%

(27/53) of the isolates presented a score ≥2.3 by method B, which is directly associated with a higher protein exposure than method A. Other authors have suggested a different cutoff point for identification, as Gautam and collaborators suggested in 2017 that a score ≥1.9 is sufficient to discriminate Bcc species. When considering the cutoff point of Gautam and coauthors [21], the use of MALDI-TOF system in our study presented 96.2% (51/53) of its identifications compatible with PCR results.

One isolate presented the following result for protocol (A) and (B), respectively: "*B. pyrrocinia*" (score 1.99) and "Member of the *Burkholderia cepacia* Complex" (score 2.03). Due to the discrepancy in this identification, the products of PCR amplification were subjected to Sanger sequencing. The results of the sequencing were blasted in GenBank, which indicated that the isolate was closely related to *B. contaminans*. Another discrepant identification by MALDI-TOF technology is the identification of *B. cepacia,* with a higher score for both protocol extractions; in a molecular assay, this isolate was confirmed as *B. cenocepacia* IIIB.

According to the literature, some species may present higher rates of incorrect identification by MALDI-TOF, such as *B. contaminans* and *B. cepacia* [25*,* 19]. The incorrect identifications are due to the formation of similar or almost equal spectra, due to the high phenotypic and genotypic similarity between Bcc species [6*,* 8]. Furthermore, some of these failures may be associated with a lack of spectra such as the case of newly cataloged bacteria. Therefore, the constant updating and expansion of databases and software can minimize problems with the identification of some microorganisms using MALDI-TOF [26*,* 27*,* 19*,* 28]. Fehlberg and coauthors [19] reported that MALDI-TOF was not able to differentiate any *B. contaminans* isolates tested, in addition to presenting the unsatisfactory identification of *B. cepacia* (77.7%). Wong and coauthors [28] also found no agreement in the identification of these isolates. Although works in the literature were shown to be successful with an in-house database for the identification of *B. contaminans* in MALDI-TOF, it cannot be used yet in a database of clinical diagnosis [29].

It is estimated that about 30% of CF patients will be colonized by Bcc and most infections are caused by *B. cenocepacia* and *B. multivorans* [30]. Those two species together account for 85-97% of cases of CF airway infection with Bcc, although other members of Bcc may also be associated with chronic infections [2]. Our epidemiological data are similar to those of another study conducted previously in the same institution [30], which indicated that 73.6% (39/53) of the isolates of Bcc corresponded to *B. cenocepacia*. In the same way as Lutz and coauthors [30], we found a prevalence of *B. cenocepacia* IIIB species when compared of *B. cenocepacia* IIIA. Notwithstanding the fact that there are no recent studies of epidemiology of genomovar of *B. cenocepacia* species, we believe that this prevalence remains the same today.

Molecular techniques have been used as reference methods for bacteria identification, but these techniques require a highly qualified workforce and demand time to perform. Despite the high cost required for the acquisition of the MALDI-TOF equipment, the reagents needed for identification using this system are usually of very low cost (approximately USD 0.2 per isolate). Moreover, MALDI-TOF allows the rapid identification (approximately 3 minutes) of bacteria and presents high accuracy.

CONCLUSION

MALDI-TOF proved to be very efficient to differentiation of *B. cenocepacia* from other species of *Burkholderia cepacia* complex. Therefore, we suggest that MALDI-TOF can be use in routine laboratories to identify the main species of Bcc, as this technique is a feasible and presents low cost of reagents.

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