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Matrix-assisted laser desorption/ionization time-of-flight MS for the accurate identification of *Burkholderia cepacia* complex and *Burkholderia gladioli* in the clinical microbiology laboratory

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Abstract

Introduction. *Burkholderia cepacia* complex (Bcc) bacteria, currently consisting of 23 closely related species, and *Burkholderia gladioli*, can cause serious and difficult-to-treat infections in people with cystic fibrosis. Identifying *Burkholderia* bacteria to the species level is considered important for understanding epidemiology and infection control, and predicting clinical outcomes. Matrix-assisted laser desorption/ionization time-of-flight MS (MALDI-TOF) is a rapid method recently introduced in clinical laboratories for bacterial species-level identification. However, reports on the ability of MALDI-TOF to accurately identify Bcc to the species level are mixed.

Aim. The aim of this project was to evaluate the accuracy of MALDI-TOF using the Biotyper and VITEK MS systems in identifying isolates from 22 different Bcc species and *B. gladioli* compared to *recA* gene sequencing, which is considered the current gold standard for Bcc.

Methodology. To capture maximum intra-species variation, phylogenetic trees were constructed from concatenated multi-locus sequence typing alleles and clustered with a novel k-medoids approach. One hundred isolates representing 22 Bcc species, plus *B. gladioli*, were assessed for bacterial identifications using the two MALDI-TOF systems.

Results. At the genus level, 100 and 97.0% of isolates were confidently identified as *Burkholderia* by the Biotyper and VITEK MS systems, respectively; moreover, 26.0 and 67.0% of the isolates were correctly identified to the species level, respectively. In many, but not all, cases of species misidentification or failed identification, a representative library for that species was lacking.

Conclusion. Currently available MALDI-TOF systems frequently do not accurately identify Bcc bacteria to the species level.

INTRODUCTION

Burkholderia cepacia complex (Bcc) bacteria are a group of at least 23 closely related species of Gram-negative bacteria, most of which are known to be capable of causing chronic lung infections in people with cystic fibrosis (CF) [1–4]. Compared to other pathogens in CF, patients with Bcc infections experience poorer outcomes, with longer hospital stays, increased risk of death and, with some species, adverse outcomes following lung transplantation [5, 6]. Identifying Bcc bacteria to the species level is therefore critical to understanding epidemiology and the effectiveness of infection control precautions, assessing for lung transplant – as some species are associated with adverse outcomes post-transplant – and guiding clinical treatment. The identification of *Burkholderia* to the species level typically necessitates clinical microbiology laboratories to send bacterial isolates to referral laboratories which use

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Abbreviations: Bcc, *Burkholderia cepacia* complex; CF, cystic fibrosis; IVD, in-vitro diagnostic; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight MS; MLST, multi-locus sequence typing; RUO, research-use only.

Two supplementary tables are available with the online version of this article.

specialized molecular assays for species identification. Accurate identification to the species level is commonly achieved using *recA* gene sequencing, as 16S rRNA gene sequences are insufficiently discriminatory within the Bcc [1, 7].

Matrix-assisted laser desorption/ionization time-of-flight MS (MALDI-TOF) has emerged as a quick, sensitive and inexpensive technique for the identification of microorganisms [8, 9]. As such, many clinical laboratories have adopted the technique as their primary method for bacterial identification [8, 10, 11]. A characteristic spectrum is generated in MALDI-TOF by irradiating a bacterial sample co-crystallized with a matrix and a species identification is achieved when this spectrum is matched to reference spectra contained in a library [9, 12, 13] .

Despite its implementation in clinical laboratories for identifying many species of bacteria, the ability of MALDI-TOF to reliably differentiate species of Bcc bacteria remains a concern. Studies have shown that genus-level identification is generally reliable, with reports of 98–100% accuracy for Bcc samples. Accuracy declines for species-level identification, with studies reporting overall values ranging from 70 to 83% [10, 14–17]. However, these studies investigated between two and 12 species of Bcc out of the current 23 species that comprise the Bcc, leaving many species untested. The reliability of MALDI-TOF in identifying *Burkholderia* bacteria is potentially impaired by the emergence of many new taxonomic Bcc species in recent years as well as the phenotypic overlap between some species within the complex [2–4, 18–21]. Therefore, this study aimed to evaluate the accuracy of MALDI-TOF using the two leading commercial platforms, namely the Biotyper system (Bruker Daltonics) and the VITEK MS system (bioMérieux), including their research databases, in identifying a variety of *Burkholderia* species. By including a wide range of Bcc species, we also sought to determine if any of the species recently added to the complex (for which there may not be reference spectra) would be confidently misidentified as other members of the Bcc.

METHODS

Isolate selection

We selected 100 isolates from 23 species (we had no representatives of *Burkholderia catarinensis* available). In order to identify representative isolates from each species, genetic analysis of published multi-locus sequence typing (MLST) sequence data was undertaken following phylogenetic tree generation and cluster analysis.

Phylogenetic trees were reconstructed from concatenated sequence data for the seven MLST alleles in the MLST typing scheme for 23 *Burkholderia* species, which were extracted from the PubMLST database [22, 23]. The data were filtered for isolates with identical strain types within the same country, except for Canadian isolates. *Burkholderia pseudomallei* isolate K96243 was also included in the dataset to act as a root for the phylogenetic tree. Sequences were then aligned using MAFFT (Multiple Alignment using Fast Fourier Transformation) v7.271 [24], with automatic settings for appropriate alignment strategy selection. Phylogenies were inferred with RAxML v8.2.4 [25] using the GTR-GAMMA model of rate heterogeneity with 100 bootstraps.

Cluster analysis was performed on trees. First, branch length distance matrices were calculated for the best-scoring maximum-likelihood trees using the 'distTips' function with the patristic method from the R package 'adephylo' v1.1–7 [26]. The *B. pseudomallei* isolate was filtered out from the clustering analysis. K-medoids clustering, using the 'pam' function from the R package 'cluster' v2.1.0 [27], was performed on the distance matrices to group isolates within a species according to genetic similarity (Fig. 1). Euclidean distance was the metric used for calculating dissimilarity. Isolates were clustered at k=5 and optimal k as determined through the 'fviz_nbcluster' function from the R package 'factoextra' v1.0.5 [28] using the elbow method. Following clustering, isolates from each species phylogeny were labelled according to the cluster, rooted to *B. pseudomallei* and visualized using the R package 'ggtree' v1.16.2 [29] (Fig. 2).

For each species, genetically representative isolates were chosen by identifying an isolate from each cluster as guided by the cluster analysis, with the higher of either the optimal k or k=5. Guided by the availability of the isolates from each cluster within our culture collection, the 100 isolates selected represent 79 clusters (Table S1, available in the online version of this article). There may have been multiple isolates selected from a given cluster if the isolates were deemed genetically different enough based on relative branch lengths on the phylogenetic tree. As such, typically four or five isolates were attained for each species, except for *Burkholderia paludis*, *Burkholderia puraquae* and *Burkholderia ubonensis*, for which we did not have enough available isolates (Table S1).

recA identification

Isolates chosen for analysis had their species identity previously established through species definition studies [2, 4, 20, 30–33] and submissions of clinical samples to the Canadian *Burkholderia cepacia* Complex Research and Referral Repository (CBCCRRR) [34]. Sequencing was repeated for isolates where discrepancies existed between the results from *recA* gene sequencing and MALDI-TOF MS. For sequencing, DNA was extracted either using Bio-Rad Instagene or by boiling extraction. PCR amplification of the *recA* gene was performed as described by Spilker *et al.* [35]. The amplicons were purified using the DNA Clean and Concentrator-5 kit (Zymo Research) and sequenced with the same *recA* primer used for PCR. Sequences were queried against the PubMLST database for species-level identification [22, 23].

MALDI-TOF MS identification

Isolates were grown on 5% Columbia sheep blood agar (Oxoid) plates at 35 or 37 °C for 18–24 h. For the Biotyper system, a single colony of bacteria was spotted in duplicate

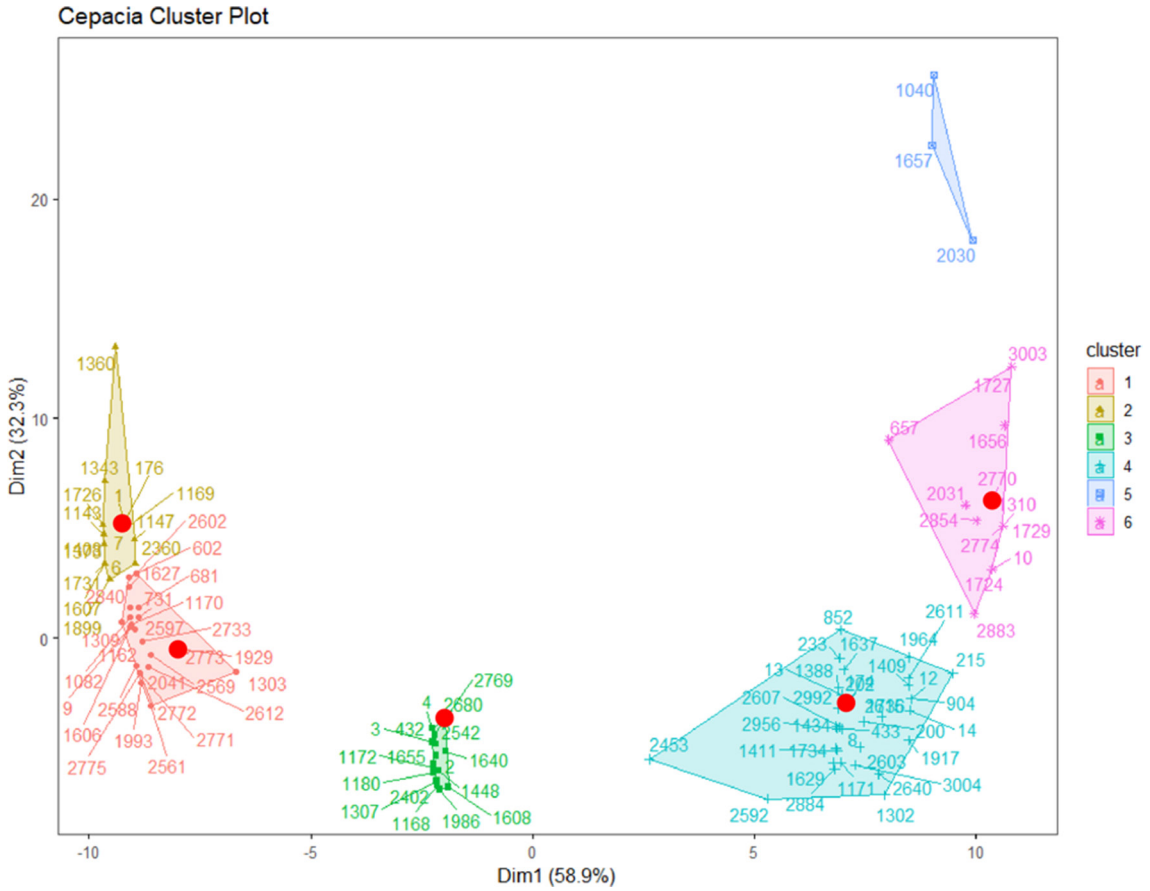

Fig. 1. K-medoids cluster plot for *Burkholderia cepacia sensu stricto*. Isolates were obtained from the PubMLST database, whereby MLST sequences were aligned using MAFFT, inputted into RAxML at 100 bootstraps and analysed for branch length distances using distTips. Distances were used to aggregate the isolates at *k*=6, which was determined through the optimal *k*-clusters using the elbow method. Numbers displayed represent the PubMLST ID for an isolate. Red dots represent the isolates selected as genetic representatives from each cluster.

onto a stainless steel MALDI target, overlaid with 1 µl of Bruker HCCA matrix (saturated solution of α-4-cyano-hydroxycinnamic acid in 50% acetonitrile and 2.5% tri-fluor-acetic acid solution) and air-dried. Similarly, for the VITEK MS system, a bacterial colony was spotted, without replicate, onto the target, overlaid with 1 µl of VITEK MS-CHCA matrix (identical to HCCA) and air-dried, or with the addition of an extraction step through treating the colony with formic acid before adding matrix. Both the Biotyper and the VITEK MS systems were calibrated and had their respective quality control standards analysed as per the manufacturers' guidelines. Samples were run using the MALDI Biotyper system on the Bruker Daltonik Microflex LT instrument and the VITEK MS research-use only (RUO) version 4.15 and in-vitro diagnostic (IVD) version 3.2 systems on the bioMérieux instrument. The libraries used for identification of the mass spectra were the MBT 7854 MSP library on the Biotyper system and the SARAMIS and Knowledge Base databases on the RUO and IVD systems, respectively. For the Biotyper system, successful genus- and species-level identification were defined following manufacturer guidelines as the highest-scoring match above 1.7 for genus and 2.0 for species where all other IDs yield the same genus or there is a difference of greater than 0.2 from the next best species match, respectively. We additionally evaluated the performance of this machine by accepting results based on the highest scoring match with an overall score of greater than 2.0, which we termed the 'first hit' criterion. For the VITEK MS system, confident genus- and species-level identification were defined as a genus and species result scoring >99% on the Knowledge Base database, respectively. Isolates scoring below this threshold would be re-analysed using the SARAMIS database, also using a >99% threshold.

Statistical analysis

To compare the results of the two MALDI-TOF systems, the Wilcoxon signed rank test with continuity correction was applied to both the genus and the species data using GraphPad Prism V6. Species-level comparisons were made

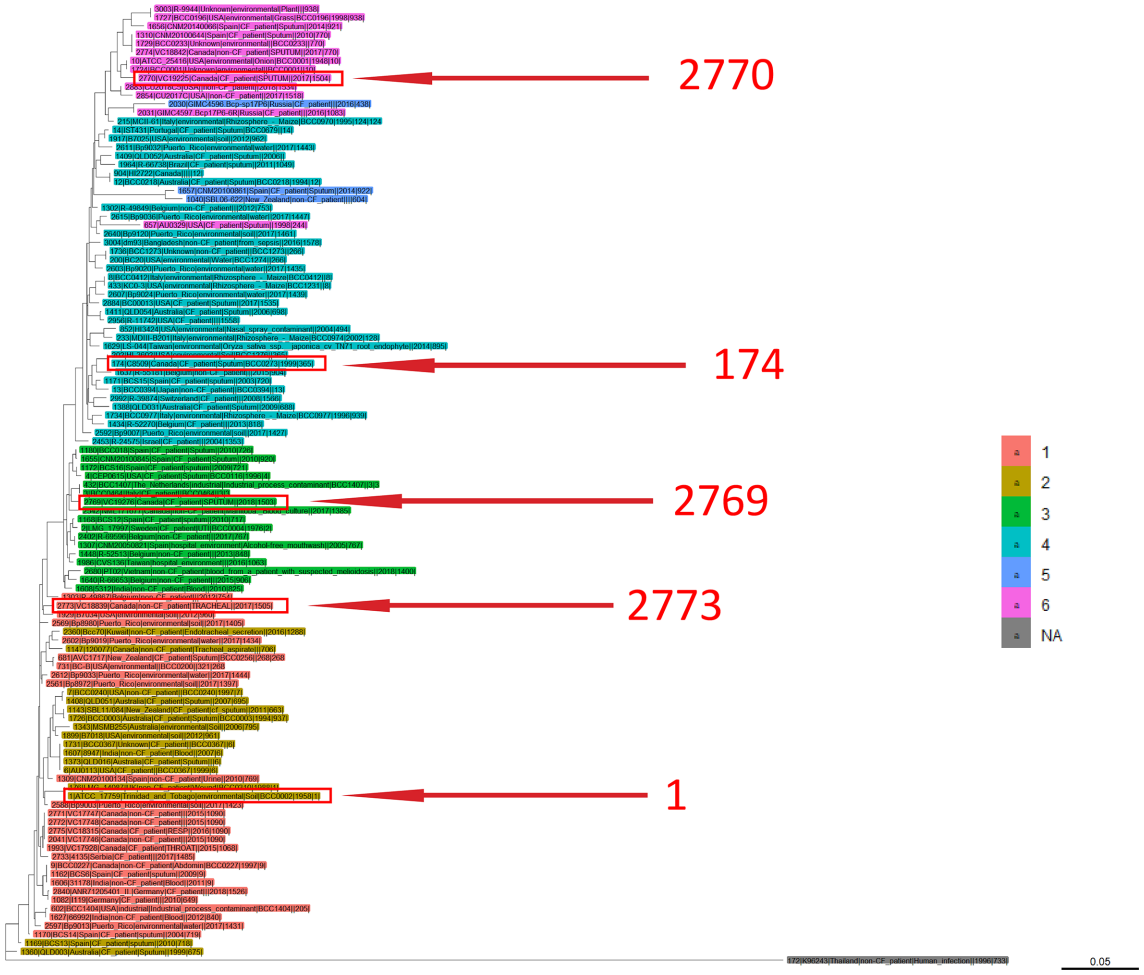

Fig. 2. Clustered phylogenetic tree for *Burkholderia cepacia*. Isolates were obtained from the PubMLST database, whereby MLST sequences were aligned using MAFFT, inputted into RAxML at 100 bootstraps and analysed for branch length distances using distTips. Distances were used to aggregate the isolates at *k*=6, which was determined through the optimal *k*-clusters using the elbow method. Isolates are coloured according to their cluster and were visualized on ggtree. Red arrows and numbers denote the isolates selected from each cluster as a genetic representative. The first number listed in annotations is the isolate's respective PubMLST ID. Scale bar represents substitutions per site

for all 100 isolates analysed in the study as well as for only the isolates with reference spectra.

RESULTS

Identification of *Burkholderia* isolates by the Bruker Biotyper MALDI-TOF system

At the genus level, 100% of the identities generated by the Biotyper system were reported as *Burkholderia* (Tables 1 and S2). Using the manufacturer guidelines for confident species-level identification (a score of over 2.0 and a minimum 0.2 difference to the next hit), 26.0% of the isolates analysed by the Bruker Biotyper had identities that matched *recA* species ID results (Table 1). For 14/23 species included in the study, no accurate species identification was obtained; however, eight of these species were lacking reference spectra within the MBT 7854 MSP library used for identification. Only two species of *Burkholderia* bacteria, *Burkholderia gladioli* and *Burkholderia dolosa*, had all their isolates correctly identified to the species level by the Biotyper system. The two most common Bcc species, *Burkholderia multivorans* and *Burkholderia cenocepacia*, yielded 80 and 50% accurate identification, respectively. When the score difference threshold was disregarded using the 'first hit' criterion, the overall concordance between the MALDI-TOF and *recA* results increased to 57.5% (Table S2). Under this criterion, species which consistently produced failed identifications were narrowed down to 8/23 species. Meanwhile, the number of species for which all isolates were confidently identified increased to include: *B. cenocepacia*, *Burkholderia ambifaria* and *Burkholderia vietnamiensis*. Regardless of the two scoring criteria, the machine was unable to distinguish between *B. cenocepacia recA* subgroups IIIA and IIIB. When increasing the score threshold of the manufacturer guidelines for a confident species-level identification

Table 1. Comparison of identification results from MALDI-TOF using Biotyper, VITEK and *recA* sequencing

***recA* sequencing identification**	**No. of isolates**	**MALDI-TOF identification**			
		Biotyper		**VITEK**	
		Genus-level concordance (%)	**Species-level concordance (%)**	**Genus-level concordance (%)**	**Species-level concordance (%)**
B. ambifaria	4	100	25	100	75
B. anthina	4	100	25	100	100
*B. arboris**	4	100	0	100	100
B. cenocepacia IIIA	5	100	40	100	100
B. cenocepacia IIIB	5	100	60	80	80
B. cepacia	5	100	0	100	80
*B. contaminans**	5	100	0	100	40
B. diffusa	5	100	20	100	60
B. dolosa	5	100	100	100	80
B. gladioli	5	100	100	100	100
B. lata	5	100	0	100	60
B. latens	4	100	25	100	100
B. metallica	4	100	0	100	25
B. multivorans	5	100	80	100	100
B. paludis†*	1	100	0	100	0
B. pseudomultivorans†*	4	100	0	50	0
B. puraquae†*	1	100	0	100	0
B. pyrrocinia	5	100	0	100	80
B. seminalis†	4	100	0	100	0
B. stabilis	5	100	0	100	100
B. stagnalis†*	4	100	0	100	0
B. territorii†*	4	100	0	100	0
*B. ubonensis**	2	100	0	100	100
B. vietnamiensis	5	100	60	100	100
Concordance for all isolates		100.0	26.0	97.0	67.0
Concordance for isolates with reference spectra		100.0	34.7	99.0	81.7

*Species lacking reference spectra on the Bruker Daltonics Biotyper system.
†Species lacking reference spectra on the bioMérieux VITEK MS system.

to 2.3, confident identifications decreased from 34.7 to 18.7% for species with reference spectra, and from 26.0 to 14.0% for all species (data not shown).

Identification of *Burkholderia* isolates by the bioMérieux VITEK MS MALDI-TOF system

For the VITEK MS system, 97.0% of the isolates analysed were assigned to the genus *Burkholderia* (Table 1), with two isolates of *Burkholderia pseudomultivorans* and one of *B. cenocepacia* IIIB yielding no genus identification. At the species level, the accuracy of the VITEK MS system was higher compared to that of the Biotyper system (Wilcoxon signed rank test P=0.0102), with 67.0% of the isolates correctly and confidently identified. Isolates from 6/23 species (*B. paludis*, *B. pseudomultivorans*, *B. puraquae*, *Burkholderia seminalis*, *Burkholderia stagnalis* and *Burkholderia territorii*) did not produce any correct identification. None of these species had corresponding reference spectra in the existing

databases. Additionally, the number of species that yielded consistent correct identifications was higher at 9/23 species in comparison with the Biotyper system. The VITEK system was also unable to distinguish between *B. cenocepacia* IIIA and IIIB.

Incorrect confident MALDI-TOF identifications

The results of both instruments were also assessed to determine the accuracy of confidently identified results. Among the 28 confident species-level identifications from the Biotyper system, two isolates confidently identified as *B. cepacia* were incorrectly identified. The *recA* sequencing had identified those isolates to represent *Burkhdolderia contaminans*. However, all the other confident MALDI-TOF identifications determined by the Bruker Biotyper system corresponded to their respective *recA* results, yielding an overall concordance of 92.9% (Table 2). While the VITEK MS system was able to identify 77 isolates with high confidence, discrepancies between confident MALDI-TOF identities and *recA* results occurred with 10 isolates from *B. ambifaria*, *B. cenocepacia*,

Table 2. Comparison of confident identifications generated by MALDI-TOF using the Biotyper and VITEK systems to *recA* sequencing

Species, as identified by MALDI-TOF	Biotyper			VITEK		
	No. of isolates confidently identified	Species-level concordance to *recA*	Correct identity of misidentifications	No. of isolates confidently identified	Species-level concordance to *recA*	Correct identity of misidentifications
B. ambifaria	1	100	–	4	75	*B. diffusa*
B. anthina	1	100	–	4	100	–
*B. arboris**	0	–	–	4	100	–
B. cenocepacia IIIA/B	5	100	–	10	90	*B. territorii*
B. cepacia	2	0	*B. contaminans* (×2)	10	40	*B. contaminans* *B. metallica* (×3) *B. seminalis* (×2)
*B. contaminans**	0	–	–	2	100	–
B. diffusa	1	100	–	3	100	–
B. dolosa	5	100	–	4	100	–
B. gladioli	5	100	–	6	83.3	*B. lata*
B. lata	0	–	–	3	100	–
B. latens	1	100	–	4	100	–
B. metallica	0	–	–	1	100	–
B. multivorans	4	100	–	5	100	–
*B. paludis**†	0	–	–	0	–	–
*B. pseudomultivorans**†	0	–	–	0	–	–
*B. puraquae**†	0	–	–	0	–	–
B. pyrrocinia	0	–	–	4	100	–
B. seminalis†	0	–	–	0	–	–
B. stabilis	0	–	–	5	100	–
*B. stagnalis**†	0	–	–	0	–	–
*B. territorii**†	0	–	–	0	–	–
*B. ubonensis**	0	–	–	2	100	–
B. vietnamiensis	3	100	–	6	83.3	*B. pyrrocinia*
Concordance for all isolates (%)	28	92.9		77	87.0	

*Species lacking reference spectra on the Bruker Daltonics Biotyper system.
†Species lacking reference spectra on the bioMérieux VITEK MS system. Confident identifications are as defined by manufacturer guidelines outlined in the Methods.

B. cepacia, *B. gladioli* and *B. vietnamiensis*. For these species, *B. cepacia* yielded the lowest accuracy, at 40% correspondence (Table 2). Compared to the Biotyper system, the concordance between confident MALDI-TOF identifications and *recA* was decreased, at 87.0%. For all incorrect confident identifications, we confirmed the original species designation by repeat *recA* sequencing (data not shown).

MALDI-TOF identification results in species lacking reference spectra

Given the expanding number of species in the Bcc, we examined the results to determine if any of the species which lacked spectra in the database would confidently identify as other species that did have spectra. For the Biotyper system, eight species lacked reference spectra, of which seven did not produce confident identification for any isolates tested (Table 2). The exception was *B. contaminans* where 2/5 isolates were confidently reported as members of *B. cepacia*. For the VITEK MS system, 6/23 species lacked reference spectra. Among these, 50% of the *B. seminalis* (*n*=2) and 25% of the *B. territorii* (*n*=1) isolates yielded confident misidentifications of *B. cepacia* and *B. cenocepacia*, respectively. When removing isolates of species for which there were no reference spectra in the database, 34.7% of isolates were correctly identified by the Biotyper system and 81.7% of isolates were correctly identified by the VITEK MS system (Table 1).

DISCUSSION

In this study, we have examined the current accuracy of two widely available commercial MALDI-TOF systems – Biotyper and VITEK MS – when challenged with isolates from 23 *Burkholderia* species comprising 22/23 species as part of the Bcc, along with *B. gladioli*, the other main *Burkholderia* pathogen in CF. Using these database versions, we found that both the Biotyper and the VITEK system can generate reliable and comparable results at the genus level. At the species level, results were mixed, with concordance values ranging from 26% to 82% depending on the system and whether or not the presence of reference spectra in the spectral match database was taken into account (Table 1). Concerningly, some isolates were confidently identified as the wrong species, including one example of an isolate wrongly identified as *B. cenocepacia*.

At the genus level, both systems produced high genus-level accuracy, with 100 and 97.0% of the *Burkholderia* isolates analysed on the Biotyper and VITEK system respectively yielding the genus *Burkholderia* (Table 1). For the 3% of isolates in which a *Burkholderia* genus ID was not returned by the VITEK system, the result was a lack of an identification rather than an inaccurate one. The results from this study are consistent with the findings from previous literature indicating that MALDI-TOF is capable of correctly identifying *Burkholderia* to at least the genus level [10, 15, 16].

At the species level, neither systems accurately identified all *Burkholderia* isolates, with 26.0 and 67.0% correct identification of all isolates tested for the Biotyper and VITEK MS systems, respectively (Table 1). The VITEK MS system yielded a higher species-level concordance, partly due to a more expansive list of *Burkholderia* species in the SARAMIS and Knowledge Base database as compared to the Biotyper system. It is also notable that neither system is currently able to separate *B. cenocepacia* into its *recA* subgroups IIIA and IIIB. This may be an important distinction because while strains of both have been associated with epidemic spread, the particularly virulent strain ET-12 belongs to the IIIA subgroup [36].

Despite employing manufacturers' recommendations for confident identifications, both systems produced confident identifications for isolates (two for Biotyper and 10 for VITEK) which would have resulted in the wrong species being reported (Table 2). Of most concern were two identifications from the VITEK machine, where an isolate of *B. territorii* was reported as a *B. cenocepacia* while an isolate of *Burkholderia lata* was reported as *B. gladioli*, as validated by repeat *recA* sequencing. Both *B. cenocepacia* and *B. gladioli* have been reported as having adverse outcomes post-lung transplant [6, 37, 38] and may be considered relative or even absolute contraindications to lung transplant in some centres [39–41]. The misidentification of *B. territorii* as *B. cenocepacia* was also previously reported in two isolates examined by the Bruker instrument [17]. Both misidentifications from the Biotyper system took place in a species, *B. contaminans*, that did not have reference spectra in the spectral match database. MALDI-TOF misidentification of this species has been reported in the literature previously [10]. These results further underline concern that relatively newly described species may be wrongly reported by MALDI-TOF systems. Of further concern, 7/10 misidentifications from the VITEK system took place in isolates of species for which there were reference spectra in the spectral match database, demonstrating that the potential for misidentification is not limited to those species without reference spectra.

MALDI-TOF MS is now considered the gold standard for routine bacterial identification in most clinical microbiology laboratories [9, 42]. An extensive body of literature supports this view for many bacterial species [16, 42–44]. There are also many advantages to MALDI-TOF as a tool for bacterial identification – including speed of identification and low cost per sample of analysis [16]. Nonetheless, this and previous studies on the accuracy of MALDI-TOF for Bcc show mixed results [10, 14–16], which depend, at least in part, on the current version of the spectral matching database used by the instrument [45]. The discrepancies between different database versions may be due to the lack of reference spectra for newly described species. For the Biotyper system, extraction using formic acid may improve results; however, this would need to be tested systematically to ensure that doing so does not also increase the number of mis-identifications. Additionally, the genetic proximity of species with MLST may be a confounding factor in obtaining a confident accurate identification – we found for the Biotyper system that scores are inversely correlated with genetic distance across the MLST tree (data not shown). This suggests that improvements in

databases may bring about improvements in resolving accuracy; however it also indicates that it can be more difficult to distinguish between species that are genetically very close through peak match scores.

A strength of our study is that it includes all but one of the currently described Bcc species. However, it is also clear that there are more species of *Burkholderia* that are yet to be formerly assigned species IDs [46]. While these may be rare in CF, our data show the potential for both novel species and species already present in the spectral match database to be misidentified. Of relevance to CF, the expanding number of Bcc species is also mirrored in other genera such as *Achromobacter* and *Acinetobacter* where similarly discrepant results with MALDI-TOF on clinical isolates have been reported [47–49]. There are some limitations to this study. First, we had no isolates available for one species: *B. catarinensis*. For others that are very rare in CF, such as *B. ubononensis*, *B. paludis* and *B. puraquae*, we tested one or two isolates in total. There were also other species for which isolate availability within our collection did not permit an isolate to be picked out from each cluster (e.g. *Burkholderia arboris*). In these cases, we chose two or more isolates as genetically distinct as possible within the same cluster through comparing phylogenetic tree branch lengths.

In summary, this is the first study we are aware of that has evaluated the ability for MALDI-TOF to distinguish between 23 species of *Burkholderia* bacteria, including many of the recently described members of the Bcc. Our data extend previous studies and further show that when challenged with a broad range of Bcc isolates, neither system is suitable for *Burkholderia* species identification with current database versions, given the current accuracy. Conceivably, future spectral match databases or the development of custom databases specific for *Burkholderia*, incorporating reference spectra for all Bcc species, may improve the ability of commercial systems to identify Bcc. Nonetheless, extensive studies, comprising many isolates for each species, as well as other isolates from currently undescribed species, will be required to confidently use this technology as a primary means of speciation for Bcc.

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Conflicts of interest
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