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The classical *Bordetella* species and MALDI-TOF technology: a brief experience

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Abstract

Purpose. The aim of this work was to evaluate and optimize the identification of *Bordetella pertussis*, *Bordetella parapertussis* and *Bordetella bronchiseptica* (usually known as the classical *Bordetella* species) using Bruker Biotyper matrix-assisted laser desorption ionization/time-of-flight mass spectrometry (MALDI-TOF MS).

Methodology. A set of 106 previously characterized clinical isolates was used. The results were interpreted according to the manufacturer's recommendations and, in addition, a new score value cutoff was used for species identification. Further, the 10 % rule (previously adopted by other authors) and the new 5 % breaking point (proposed in this work) were evaluated in order to optimize identification rates.

Results/Key findings. Our results suggest that it is possible to distinguish different species of the classical *Bordetella* species by following a simple algorithm without additional testing being required.

Conclusion. MALDI-TOF might be a reliable tool for the identification of this group of bacteria when a combination of cutoff scores is used. This procedure allows us to increase the identification rates for the classical *Bordetella* species significantly; however, more studies will be required to determine the applicability of the method to other difficult-to-distinguish organisms.

INTRODUCTION

Bordetella pertussis (Bp), an exclusive human pathogen, is the main causative agent of pertussis, an acute respiratory and vaccine-preventable disease [1]. There are additional members of the family, such as *Bordetella bronchiseptica* (Bb), *Bordetella parapertussis* (Bpp) and *Bordetella holmesii* (Bh), which are also associated with respiratory tract infections. Other members of the genus are less known; however, infections due to these organisms have been reported, especially in immunocompromised hosts [2]. Pertussis disease, also known as whooping cough, is divided into three stages: the catarrhal, the paroxysmal and the convalescence stage. This is very important to consider because laboratory diagnostics tools are strictly related to these stages.

Despite its low sensitivity and slow growth, culture remains the gold standard for pertussis diagnosis [3]. Nonetheless, real-time polymerase chain reaction (RT-PCR) has become the recommended and most commonly used method in the laboratory for the diagnosis of whooping cough [4]. Further, it has the advantage of being applied directly on the clinical sample (nasopharyngeal aspirate/nasopharyngeal swab). The remaining tool for pertussis diagnosis is serology, but standardization of this technique is still in process and it can only be used in adolescents and adults [5].

In the last few years the introduction of matrix-assisted laser desorption ionization/time-of-flight mass spectrometry (MALDI-TOF MS) into the microbiology laboratory has been shown to be useful for bacterial identification [6]. In brief, this new technology compares protein profiles by generating spectra based on the *m*/*z* ratio and then it compares them against a database.

As mentioned previously, culture is the gold standard for pertussis (Bp grows optimally on either Regan-Lowe or Bordet-Gengou agar and can be differentiated based on its growth). It is 100 % specific, but has low sensitivity (12–60 %) and any suspicious isolate must still be confirmed either by biochemical characteristics or molecular techniques.

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Abbreviations: Bb, *Bordetella bronchiseptica*; Bh, *Bordetella holmesii*; Bp, *Bordetella pertussis*; Bpp, *Bordetella parapertussis*; CC, consistency criteria; MALDI-TOF MS, matrix-assisted laser desorption ionization/time-of-flight mass spectrometry; RT- PCR, real-time polymerase chain reaction.

Compared with conventional phenotype- or PCR-based identification, MALDI-TOF shows a rapid turnaround time, low sample volume requirements and modest reagent costs [7]. With these efficiencies, MALDI-TOF could be a relevant substitute for the current methodology in species-specific classification of *Bordetella* obtained from culture.

However, this new technology can erroneously identify closely related microorganisms, for example mistaking *Streptococcus pneumoniae* for *Streptococcus mitis*, *Escherichia coli* for *Shigella* spp. [8]. The same problem occurs with the classical *Bordetella* species; therefore, distinguishing their species by MALDI-TOF using proprietary algorithms can be difficult.

Usually the main criteria used to analyse the result of the spectral database are score values (score values range from 0.0 to 3.0). Most clinical microbiology laboratories use score thresholds that are suggested by the manufacturer [9], although other cutoff values may be used to optimize and improve bacterial identification, even for yeast [10].

On the other hand, interpretation of the results is not usually as simple, especially when incorrect species with high score values are reported among the top 10 results. Briefly, for each sample, the MALDI-TOF software reports the 10 best matches between the unknown sample data and the reference database. Results are also categorized based on the identification consistency criteria (CC) included in the MALDI Biotyper software (v3.0). A '10 % rule' was previously used to address the multiple matches among the top 10 results [11–14].

There is currently a lack of publications in the literature for the application of MALDI-TOF MS to *Bordetella* spp. and this study evaluated the performance of MALDI-TOF in combination with a simple algorithm to improve the identification of classical *Bordetella* species using this platform.

METHODS

In this study, a total of 106 clinical isolates of the classical *Bordetella* species that had previously characterized by molecular methods and biochemical tests (94 Bp, 5 Bpp and 7 Bb) and were collected at the INEI-ANLIS 'Dr Carlos G. Malbrán and Hospital de Clínicas José de San Martín, Universidad de Buenos Aires, Argentina, were analysed using MALDI-TOF technology. Regan-Lowe agar was used as the isolation media. The identification was considered to be correct when the molecular method, biochemical test and MALDI-TOF agreed at the species level. When some of the methods yielded discordant results, real-time PCR was considered to be the gold standard identification method for this study.

DNA extraction

Nucleic acids were extracted from a bacterial suspension using the QiaAmp DNA minikit (Qiagen, Hamburg, Germany) according to manufacturer's instructions.

Real-time PCR

We used a combination of PCR assays. First, we employed a multiplex RT-PCR assay, which included three targets sequences: IS*481* (20–100 copies/cell in Bp, 5–8 copies/cell in Bh and <5 copies/cell in Bb), hIS*1001* (3–5 copies/cell in Bh) and pIS*1001* (20–23 copies/cell in Bpp). Then, we employed a second singleplex RT-PCR for pertussis toxin subunit S1 [15, 16]. Although it is not possible to identify *Bordetella bronchiseptica* directly from the triplex assay, its presence can be presumed by using a *ptxS1* assay.

Both PCRs were performed using the Applied Biosystems 7500 Fast Real-Time PCR System (Life Technologies Corp.) and, in addition, Applied Biosystems TaqMan Gene Expression Master Mix (Life Technologies Corp.).

Real-time PCR interpretation

According to the previously described targets, the results for species identification are summarized in Table 1. Briefly, a positive reaction for I*S481* and *ptxS1* is conclusive for *B. pertussis*. When both pIS*1001* and *ptxS1* are positive, it is confirmatory for *B. parapertussis*. Nevertheless, a positive reaction for *ptxS1* and a negative one for any of the other targets is suspicious for *B. bronchiseptica*, in which case, additional studies should be performed [17].

Biochemical identification

All isolates were identified using a biochemical test proposed in the literature [18] and this is summarized in Table 2.

Sample processing for identification by MALDI-TOF

The analysis was carried out using the Bruker Daltonics MicroFlex LT instrument version 3.4 (Bruker Daltonics, Germany). The whole-cell direct transfer method was carried out based on the manufacturer's recommendations. Briefly, a colony of bacteria was applied onto the steel MALDI plate, then 1.0 µl of HCCA matrix (a-cyano-4-hydroxy-cinnamic acid) (Bruker Daltonics) was added to the sample and allowed to dry. Immediately after, the plate was loaded into the instrument and analysed using MALDI-TOF software (MALDI Biotyper 3.1). The spectra were analysed using the MALDI Biotyper Library (MBT Compass Library, DB-5989 MSP, #1829023). This database contained 9 spectra for *B. bronchiseptica*, 11 spectra for *B. parapertussis* and 10 spectra for *B. pertussis*. This database also contained spectra for the other species of *Bordetella*, except *Bordetella ansorpii* [19].

MALDI-TOF MS data interpretation

Different approaches were evaluated for the interpretation of results. First, there were the cutoffs recommended by the manufacturer, which are calculated as follows. The spectrum of the unknown test bacteria, acquired through the MALDI Biotyper software, is transformed into a peak list. Using Bruker's proprietary algorithm, this peak list is compared to reference peak lists of organisms in the reference database and a score value between 0.00 and 3.00 is

calculated. The higher the score value, the higher the degree of similarity to a given organism in the reference database. A score value of ≥2.00 can be considered to be identification at the species level and is shown in green. A score value of between 1.70 and 1.99 represents a reliable genus-level identification, and is shown in yellow. A score <1.699 is completely unreliable and is shown in red. After the acquisition of the spectral data has been completed, a Run Results report is generated. The resultant report for each sample shows the 10 best matches along with the respective matching score.

The second approach to be reviewed was consistency categories (CCs). In addition to the score, the sample match result is also given a CC letter. This data parameter gives an indication of consistency quality. The categories are rated as Species Consistent (A), in which the best match is classified as green, and further green matches are of the same species as the first one. Further yellow matches are at least of the same genus as the first one. The second category is denominated Genus Consistent (B); in this case, the best match is classified as green or yellow. Further green or yellow matches have at least the same genus as the first one; further, different species of the same genus with score values of ≥2.0 are present in the 10 best matches. The last category is denominated No Consistency (C). In this case the top best match is classified as red.

Considering the aforementioned, we decided to modify the B consistency category, proposing instead a sub-classification into Consistency B Major (matches scoring >2.0 are not the same species) and Consistency B Minor (top match score 1.99–1.7). Consistency B Major represented an issue of concern, because it indicated uncertainty regarding a true identification. To address this complicated situation we applied the previously mentioned 10 % rule and a new 5 % breaking point (5 % BP).

Statistical analysis

The association between categorical variables was analysed by the chi-square test; in cases in which the sample sizes were small, Fisher's exact test was used. In all cases, a significance level of 5 % was considered. Statistical calculations were performed with Epi Info 7 (Center for Disease Control and Prevention, Atlanta, GA, USA).

RESULTS

The results were analysed according to different approaches: score values, consistency category, top 10 match variability, modification of the standard cutoff, and finally the 10 % rule and the 5 % breaking point.

Data interpretation according to score values

Of the 106 isolates previously characterized by RT-PCR and biochemical tests, 75 (70.75 %) presented a score value of >2.0. On the other hand, 30 isolates (28.3 %) scored between 1.70 and 1.99, and only 1 isolate showed a score of <1.699.

Concordance by score values

All isolates (106/106, 100 %), independently of the score value, showed excellent agreement with the other two methodologies used, but this agreement is only valid if the top match is contemplated. Moreover, only isolates with the score <1.699 had very good concordance with the RT-PCR and biochemical test employed when the top match was considered. This result made us ponder whether the score values proposed by the manufacturer might be in some measure conservative, and whether a reduction of the species-level threshold could be an alternative to optimize classical *Bordetella* species identification.

Data interpretation and concordance according to CC

The consistency category A results for isolates in MALDI-TOF showed excellent species-level concordance with RT-PCR and the biochemical test (66/66, 100 %). As mentioned above, consistency category B was classified into CC B Major and CC B Minor. Of the 106 isolates evaluated, 9 (9 %) (3 Bp, 5 Bb and 1Bpp) were classified as CC B Major; therefore, the MALDI-TOF results could not be informed without applying an additional test (molecular or biochemical) to confirm species identification. But again, they had very good concordance with the RT-PCR and biochemical test employed if the top match was considered and no complementary test was required. The data also showed that *Bordetella bronchiseptica* was the species with the most CC B Major isolates (5/7, 71 %) and this misidentification was always in relation to *B. parapertussis*. The MALDI-TOF results for CC B Minor isolates also showed very good agreement (30/30, 100 %) when the results were compared with those for the other two techniques. However, as we stated above, this agreement was only found when the top match was considered. In Table 3 the results are summarized according to the score value and CC for all species evaluated.

Top 10 match variability

As mentioned previously, the Bruker MALDI-TOF software reports the 10 best matches for each isolate from the highest score value to the lowest. Most publications do not contemplate all 10 results; they only address the first 2 best matches. Given this, we proposed the term 'critical mismatch', which is related to the consistency category B Major. Briefly, this term is used to refer to the presence of multiple species with a score value >2.0 in the top 10 best matches that differ from the top match identification.

Table 1. Interpretation of real-time PCR results

Targets	*B. pertussis*	*B. parapertussis*	*B. bronchiseptica*
IS*481*	+	−	−
pIS*1001*	−	+	−
ptxS1	+	+	+

Table 2. Differential characteristics of *Bordetella* species

Characteristic	*B. pertussis*	*B. parapertussis*	*B. bronchiseptica*
Oxidase	+	−	+
Urease*	−	+	+
Motility	−	−	+

*It can take 24 h to obtain a positive urease test result for Bpp, whereas for Bb it takes 4 h.

Our findings showed that only 37/106 (35 %) of the isolates did not present discrepant results between the top match and the top 10 matches; of these isolates, 27/37 (73 %) belonged to consistency category A (25 Bp and 2 Bpp) and 10/37 (27 %) belonged to consistency category B minor (all belonged to Bp). Only 9 % (9/106) presented critical mismatches, which is coincident with the consistency category B Major. For the remaining 60/106 (57 %) isolates, the top 10 best matches contained at least 1 species mismatch, but with score value <2.0. Of these 60 isolates, 39 were category consistency A (36 Bp, 2Bb and 1 Bpp), 20 were consistency category B minor (19 Bp and 1 Bpp) and 1 was consistency category C (Bp).

Concordance according to top 10 match variability

The species-level concordance between isolates identified by MALDI-TOF and the other methodologies was excellent. Moreover, for all the isolates studied, the first mismatch or critical mismatch only appeared only in the third or fourth position of the top 10. As mentioned previously, all of these data suggest that the manufacturer's recommended thresholds (score value and consistency categories) could be modified in order to improve classical *Bordetella* species identification.

The 10 and 5 % discriminatory 'rules'

In order to optimize the level of identification, when multiple species mismatches (especially critical mismatches) were among the top 10 best matches, the 10 % rule and the 5 % breaking point mentioned above were applied to the first species discrepancy that appeared in the top 10 list. In brief, for the 10 % rule, we applied Khot *et al.*'s 2012 criteria, according to which 'any species scoring >10 % below the top-scoring match may be excluded' and, using the same approach, this paper suggests the use of the 5 % breaking point, which states that, to accept the first identification of the top 10 list, a >5 % log difference between the top scoring match and the first species discrepancy should be present.

First, these rules were applied to all isolates with a score value >2.0 (including consistency category A plus consistency category B Major), and when the 10 % rule was used, 12 of 75 isolates (16 %) did not pass this cutoff. Moreover, 4/12 (2 Bp and 2 Bb) isolates belonged to consistency criteria B Major, and thus critical mismatches were present in the top 10 best matches, rendering identification by MALDI-TOF not fully reliable, and making it necessary to perform additional tests to confirm the species. On the other hand, when the empirical 5 % breaking point was used, 75 of 75 isolates passed this cutoff, without additional testing being required (*P*-value 0.043). When we applied this analysis solely to the 9 isolates classified as consistency category B Major, the result was significant (*P*-value 0.001).

Finally, if consistency category B Minor is analysed, when the 10 % rule was used, 25 of 30 isolates passed the cutoff; but when the 5 % breaking point was applied, 30/30 passed the cutoff (*P*-value 0.002) According to the data obtained, this 5 % discriminatory rule was able to resolve the discrepancy among the B Major consistency category isolates, and thus the critical mismatches. Moreover, it can be used to optimize isolates classified as B minor.

Modification of standard score values

Another approach to be reviewed was the modification of the manufacturer's score values. We proposed a reduction of the standard score value for species-level identification (from 2.0 to >1.7) in order to optimize classical *Bordetella* species identification in line with the results obtained.

When the standard cutoff suggested by the manufacturer was used, 75 out of 106 (71 %) isolates yielded a matching species-level identification between MALDI-TOF and the other methodologies employed (as mentioned above), but when the score value was downgraded to >1.7 for species-level identification, 105 out of 106 isolates (99 %) gave a concordant species-level identification (*P*-value <0.001). Therefore, when we changed the threshold from 2.0 to 1.7, the identification rates at the species level increased to 40 % and the results were statistically significant.

DISCUSSION

Pertussis diagnostics can be approached by different methodologies, but the sensitivity of all of them depends on

Table 3. Results according the score value and CC for all of the species evaluated

Species	Score values			Consistency categories			
	>2.0	1.70–1.99	<1.699	A	B Major	B Minor	C
Bordetella pertussis	64 (68 %)	29 (31 %)	1 (1 %)	61 (65 %)	3 (3 %)	29 (31 %)	1 (1 %)
Bordetella parapertussis	4 (80 %)	1 (20 %)	0	3 (60 %)	1 (20 %)	1 (20 %)	0
Bordetella bronchiseptica	7 (100 %)	0	0	2 (29 %)	5 (71 %)	0	0

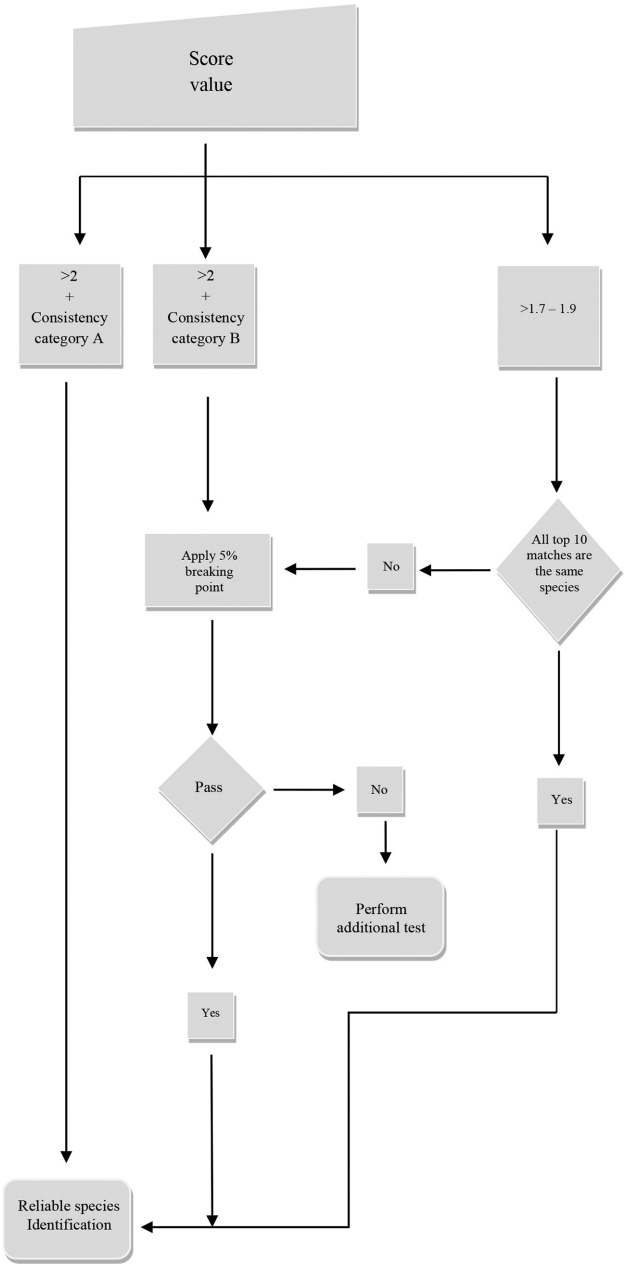

Fig. 1. Algorithm proposed for the identification of the classical *Bordetella* species by MALDI-TOF.

several factors, as previously mentioned. However, culture remains the gold standard and bacteria are usually identified by biochemical characteristics or molecular methods. However, in the last few years MALDI-TOF technology has erupted into microbiology laboratories and changed the paradigm of bacteria identification. In the present study, we evaluated the performance of this new methodology to identify the classical *Bordetella* species, using 106 previously characterized isolates, in order to consider it as an alternative tool for identifying this microorganism within minutes instead of hours or days.

MALDI-TOF results are usually interpreted according to the manufacturer's recommendations (score values and consistency category) and most of the time these criteria are sufficient for the majority of clinical microbiology laboratories for routine use. However, the results are not always easy to interpret, especially when different species with high score values of the same genera are displayed among the top 10 best matches in the MALDI-TOF list. To address this situation, one solution proposed by Khot *et al.* [12] was the application of the 10 % differential rule, as previously described, and this approach was evaluated in the present work in order to consider it as a potential solution for discrepant results between different species of *Bordetella*. In addition, we proposed, the 5 % breaking point, with the intention of giving more robustness to the identification obtained with the 10 % rule.

Some publications have suggested that increasing the reference database and even applying the extraction method may improve identification rates. We performed these two approaches (data not published) and, despite the fact that we improved the score values, we also continued to experience mismatching identifications in the top 10 report.

According to the data analysed, the use of MALDI-TOF MS for *Bordetella* species identification seems useless when compared with RT-PCR: only 66 of 106 isolates (62 %) showed excellent species-level agreement without the need for additional testing when the manufacturer's recommendations were used. Only nine isolates presented significant species mismatches, and additional tests were needed (such as oxidase, urease, motility or molecular tests) to confirm species identification. However, 30 isolates had score values between 1.7 and 1.9, as assessed in the genus consistency category (according to the manufacturer's consistency criteria), and in all of these we found that the first mismatch appeared in the fifth position of the top 10 list. This led us to infer that perhaps the score cutoff suggested by the manufacturer was somewhat conservative.

In view of this, we also evaluated the possibility of reducing the standard species score values, as proposed by Van Veen *et al.* [20], who had increased identification rates for fastidious Gram-negatives rods; similar observations were made before by Bizzini *et al.* [21] and Szabados *et al.* [22]. Briefly, all of these studies suggest that lower species cutoff improves the identification of bacteria in general. Our study demonstrated the same results as those of the authors named above.

We acknowledge that the small number of isolates included in this study (especially for *B. parapertussis* and *B. bronchiseptica*) may have been a limitation, and more isolates need to be analysed to make a statement. In addition, it is also important to take the lack of external validation to evaluate the reproducibility of the results into consideration when analysing this work.

However, this study with MALDI-TOF technology and *Bordetella* species comprises the first report in the literature to

show a very detailed analysis of the performance of this technology with this genus. Based on our results, we suggest an optimized algorithm to increase the identification rates for *Bordetella* species, as summarized in Fig. 1.

Conclusion

MALDI-TOF might be a reliable tool for the identification of this group of bacteria when a combination of cutoff scores is used. This procedure allows us to increase the identification rates for the classical *Bordetella* species significantly; however, more studies will be required to determine the applicability of the method to other difficult-to-distinguish organisms.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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