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Rapid Differentiation of *Haemophilus influenzae* and *Haemophilus haemolyticus* by Use of Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry with ClinProTools Mass Spectrum Analysis

Jonathan H. K. Chen,[a] Vincent C. C. Cheng,[a,b] Chun-Pong Wong,[a] Sally C. Y. Wong,[a] Wing-Cheong Yam,[a] Kwok-Yung Yuen[a,c]

Department of Microbiology, Queen Mary Hospital, Hong Kong Special Administrative Region, China[a]; Infection Control Team, Queen Mary Hospital, Hong Kong Special Administrative Region, China[b]; Carol Yu Centre for Infection, The University of Hong Kong, Hong Kong Special Administrative Region, China[c]

ABSTRACT *Haemophilus influenzae* is associated with severe invasive disease, while *Haemophilus haemolyticus* is considered part of the commensal flora in the human respiratory tract. Although the addition of a custom mass spectrum library into the matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) system could improve identification of these two species, the establishment of such a custom database is technically complicated and requires a large amount of resources, which most clinical laboratories cannot afford. In this study, we developed a mass spectrum analysis model with 7 mass peak biomarkers for the identification of *H. influenzae* and *H. haemolyticus* using the ClinProTools software. We evaluated the diagnostic performance of this model using 408 *H. influenzae* and *H. haemolyticus* isolates from clinical respiratory specimens from 363 hospitalized patients and compared the identification results with those obtained with the Bruker IVD MALDI Biotyper. The IVD MALDI Biotyper identified only 86.9% of *H. influenzae* (311/358) and 98.0% of *H. haemolyticus* (49/50) clinical isolates to the species level. In comparison, the ClinProTools mass spectrum model could identify 100% of *H. influenzae* (358/358) and *H. haemolyticus* (50/50) clinical strains to the species level and significantly improved the species identification rate (McNemar's test, $P < 0.0001$). In conclusion, the use of ClinProTools demonstrated an alternative way for users lacking special expertise in mass spectrometry to handle closely related bacterial species when the proprietary spectrum library failed. This approach should be useful for the differentiation of other closely related bacterial species.

KEYWORDS MALDI-TOF, *Haemophilus haemolyticus*, *Haemophilus influenzae*, ClinProTools

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Address correspondence to Kwok-Yung Yuen, kyyuen@hkucc.hku.hk.

Haemophilus influenzae and *Haemophilus haemolyticus* are the most prevalent *Haemophilus* species colonizing the human pharyngeal cavity (1). *H. influenzae* causes various respiratory tract diseases, such as sinusitis, otitis media, and pneumonia, as well as infective exacerbations of chronic obstructive pulmonary disease and bronchiectasis (2). In contrast, *H. haemolyticus* colonizes healthy subjects at moderate prevalence and rarely causes infections in the respiratory tract (3–5).

Laboratory differentiation of the two species based on phenotypic tests can be difficult. *H. haemolyticus* and *H. influenzae* demonstrate similar colony morphologies, and both require hemin (X factor) and NAD (V factor) for growth. Hemolytic activity on

rabbit or horse blood agar was previously used as an important factor for the differentiation of these two species, but this method had been shown to be unreliable, and loss of hemolytic activity of *H. haemolyticus* after subculture can happen (1, 6, 7). Molecular diagnosis through 16S rRNA gene sequencing or PCR with specific genes, such as the *Haemophilus* protein D gene (*hpd*) or fuculose kinase gene (*fucK*), have been shown to be reliable alternatives. The prevalence of either or both of the *hpd* and *fucK* genes in *H. influenzae* can be up to 92% (8–11). However, molecular testing is expensive and technically demanding.

Recently, proteomic profiling by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS), an alternative to biochemical and molecular identification methods, has been evaluated in species differentiation for a variety of microorganisms, with promising results (12, 13). However, some closely related bacterial species could not be well differentiated by the MALDI Biotyper (Bruker Daltonics, Bremen, Germany) alone, and additional mass spectrum analyses would be required for complete resolution (14–17). Although previous studies demonstrated that MALDI-TOF MS could be used to differentiate *H. influenzae* from *H. haemolyticus*, *H. influenzae* strains from China were subsequently reported to have protein mass spectra different from those of strains isolated from other areas (18). Additional local *Haemophilus* spectrum entries to the manufacturer's reference library would be necessary to optimize the accuracy of species identification (19).

In addition to the use of MALDI Biotyper alone for bacterial identification, mass spectrum analysis through the use of the Bruker ClinProTools software (Bruker Daltonics, Bremen, Germany) has been introduced for the detection of critical biomarker mass peaks, and to develop classification models for the identification of certain phylogenetically related bacterial species in a rapid and user-friendly manner (14).

In this study, we aimed to establish a reliable rapid diagnostic method for the differentiation of *H. influenzae* and *H. haemolyticus* from clinical isolates using MALDI-TOF MS with ClinProTools mass spectrum model analysis.

RESULTS

Routine biochemical and molecular identification. All 408 *Haemophilus* species isolates were oxidase-positive Gram-negative rods demonstrating X and V factor dependence on Columbia horse blood agar (CHBA). Hemolysis was observed on CHBA within 48 h of incubation in 46 out of the 408 isolates. Of the 408 strains, 314 were *hpd* positive by PCR. Among the 94 *hpd*-negative isolates, 44 were indicated *fucK* positive by PCR. These 358 (314 *hpd* positive and 44 *hpd* and *fucK* positive) isolates were considered *H. influenzae*. The remaining 50 *hpd*- and *fucK*-negative isolates were subjected to 16S rRNA gene sequencing, and all of them were identified as *H. haemolyticus* through NCBI BLAST. These 50 *H. haemolyticus* isolates included the 46 strains showing hemolysis on CHBA.

IVD MALDI Biotyper identification alone. The stand-alone IVD MALDI Biotyper yielded concordant results for 360/408 (88.2%) *Haemophilus* strains, including 311/358 (86.9%) *H. influenzae* and 49/50 (98.0%) *H. haemolyticus* strains, compared to the routine identification method. An indeterminate result was obtained for 48/408 (11.8%) isolates, where both *H. influenzae* and *H. haemolyticus* with scores of >2.0 were included in the IVD MALDI Biotyper top 10 identification matches (Table 1). According to the standard operating procedure of the Bruker IVD MALDI Biotyper system, these 48 isolates could only be confirmed to the genus level as *Haemophilus* species, and further tests would be necessary for species confirmation.

ClinProTools model analysis. Due to the incomplete identification of the IVD MALDI Biotyper alone, the use of mass peak analysis with ClinProTools was explored for the complete solution to differentiate *H. influenzae* from *H. haemolyticus*. In ClinProTools, a classification model based on 480 spectra of *H. influenzae* or *H. haemolyticus* reference strains was developed using the support vector machine (SVM) algorithm. The classification model included 7 mass peaks in the range of 4,459.73 Da to 9,421.92 Da which demonstrated 100% recognition capability (RC) and cross validation (CV). These 7 mass

TABLE 1 Characteristics of the 48 *Haemophilus* isolates with undetermined species identification using stand-alone MALDI Biotyper[a]

	Result by:							
	Biochemical tests		Molecular tests			MALDI Biotyper		
Isolate no.	X and V factor dependent	Hemolysis	*hpd*	*fucK*	Confirmed ID	Highest score ID (Bruker score)	Other species within the top 10 IDs (Bruker score)	ClinProTools model
4	Y	N	N	P	*H. influenzae*	*H. influenzae* (2.316)	*H. haemolyticus* (2.224)	*H. influenzae*
6	Y	N	P	ND	*H. influenzae*	*H. influenzae* (2.235)	*H. haemolyticus* (2.186)	*H. influenzae*
12	Y	N	P	ND	*H. influenzae*	*H. influenzae* (2.212)	*H. haemolyticus* (2.14)	*H. influenzae*
22	Y	N	P	ND	*H. influenzae*	*H. influenzae* (2.257)	*H. haemolyticus* (2.199)	*H. influenzae*
23	Y	N	N	P	*H. influenzae*	*H. influenzae* (2.315)	*H. haemolyticus* (2.24)	*H. influenzae*
24	Y	N	P	ND	*H. influenzae*	*H. haemolyticus* (2.207)	*H. influenzae* (2.203)	*H. influenzae*
26	Y	N	P	ND	*H. influenzae*	*H. influenzae* (2.358)	*H. haemolyticus* (2.283)	*H. influenzae*
27	Y	N	P	ND	*H. influenzae*	*H. influenzae* (2.289)	*H. haemolyticus* (2.275)	*H. influenzae*
39	Y	N	N	P	*H. influenzae*	*H. influenzae* (2.227)	*H. haemolyticus* (2.185)	*H. influenzae*
42	Y	N	P	ND	*H. influenzae*	*H. influenzae* (2.233)	*H. haemolyticus* (2.107)	*H. influenzae*
44	Y	N	P	ND	*H. influenzae*	*H. influenzae* (2.382)	*H. haemolyticus* (2.244)	*H. influenzae*
56	Y	N	P	ND	*H. influenzae*	*H. influenzae* (2.392)	*H. haemolyticus* (2.234)	*H. influenzae*
57	Y	N	P	ND	*H. influenzae*	*H. influenzae* (2.277)	*H. haemolyticus* (2.194)	*H. influenzae*
64	Y	N	P	ND	*H. influenzae*	*H. influenzae* (2.314)	*H. haemolyticus* (2.254)	*H. influenzae*
70	Y	N	P	ND	*H. influenzae*	*H. influenzae* (2.357)	*H. haemolyticus* (2.273)	*H. influenzae*
78	Y	Y	N	N	*H. haemolyticus*[b]	*H. haemolyticus* (2.294)	*H. influenzae* (2.212)	*H. haemolyticus*
91	Y	N	P	ND	*H. influenzae*	*H. influenzae* (2.388)	*H. haemolyticus* (2.244)	*H. influenzae*
96	Y	N	N	P	*H. influenzae*	*H. influenzae* (2.234)	*H. haemolyticus* (2.189)	*H. influenzae*
99	Y	N	P	ND	*H. influenzae*	*H. influenzae* (2.243)	*H. haemolyticus* (2.174)	*H. influenzae*
106	Y	N	P	ND	*H. influenzae*	*H. influenzae* (2.328)	*H. haemolyticus* (2.182)	*H. influenzae*
117	Y	N	P	ND	*H. influenzae*	*H. influenzae* (2.418)	*H. haemolyticus* (2.241)	*H. influenzae*
123	Y	N	P	ND	*H. influenzae*	*H. influenzae* (2.384)	*H. haemolyticus* (2.192)	*H. influenzae*
124	Y	N	P	ND	*H. influenzae*	*H. influenzae* (2.274)	*H. haemolyticus* (2.144)	*H. influenzae*
126	Y	N	P	ND	*H. influenzae*	*H. influenzae* (2.233)	*H. haemolyticus* (2.098)	*H. influenzae*
129	Y	N	P	ND	*H. influenzae*	*H. influenzae* (2.272)	*H. haemolyticus* (2.184)	*H. influenzae*
147	Y	N	P	ND	*H. influenzae*	*H. influenzae* (2.352)	*H. haemolyticus* (2.234)	*H. influenzae*
148	Y	N	P	ND	*H. influenzae*	*H. influenzae* (2.38)	*H. haemolyticus* (2.205)	*H. influenzae*
157	Y	N	P	ND	*H. influenzae*	*H. influenzae* (2.298)	*H. haemolyticus* (2.199)	*H. influenzae*
159	Y	N	P	ND	*H. influenzae*	*H. influenzae* (2.353)	*H. haemolyticus* (2.26)	*H. influenzae*
169	Y	N	P	ND	*H. influenzae*	*H. influenzae* (2.367)	*H. haemolyticus* (2.234)	*H. influenzae*
170	Y	N	P	ND	*H. influenzae*	*H. influenzae* (2.313)	*H. haemolyticus* (2.239)	*H. influenzae*
171	Y	N	P	ND	*H. influenzae*	*H. influenzae* (2.274)	*H. haemolyticus* (2.213)	*H. influenzae*
172	Y	N	N	P	*H. influenzae*	*H. influenzae* (2.268)	*H. haemolyticus* (2.238)	*H. influenzae*
246	Y	N	P	ND	*H. influenzae*	*H. influenzae* (2.256)	*H. haemolyticus* (2.187)	*H. influenzae*
255	Y	N	P	ND	*H. influenzae*	*H. influenzae* (2.229)	*H. haemolyticus* (2.205)	*H. influenzae*
267	Y	N	N	P	*H. influenzae*	*H. influenzae* (2.205)	*H. haemolyticus* (2.085)	*H. influenzae*
277	Y	N	P	ND	*H. influenzae*	*H. influenzae* (2.259)	*H. haemolyticus* (2.205)	*H. influenzae*
278	Y	N	P	ND	*H. influenzae*	*H. influenzae* (2.418)	*H. haemolyticus* (2.339)	*H. influenzae*
284	Y	N	P	ND	*H. influenzae*	*H. influenzae* (2.282)	*H. haemolyticus* (2.236)	*H. influenzae*
324	Y	N	P	ND	*H. influenzae*	*H. haemolyticus* (2.292)	*H. influenzae* (2.289)	*H. influenzae*
336	Y	N	P	ND	*H. influenzae*	*H. influenzae* (2.37)	*H. haemolyticus* (2.22)	*H. influenzae*
340	Y	N	P	ND	*H. influenzae*	*H. influenzae* (2.386)	*H. haemolyticus* (2.288)	*H. influenzae*
348	Y	N	P	ND	*H. influenzae*	*H. influenzae* (2.333)	*H. haemolyticus* (2.272)	*H. influenzae*
359	Y	N	P	ND	*H. influenzae*	*H. influenzae* (2.269)	*H. haemolyticus* (2.207)	*H. influenzae*
360	Y	N	P	ND	*H. influenzae*	*H. influenzae* (2.379)	*H. haemolyticus* (2.186)	*H. influenzae*
364	Y	N	P	ND	*H. influenzae*	*H. influenzae* (2.346)	*H. haemolyticus* (2.191)	*H. influenzae*
369	Y	N	P	ND	*H. influenzae*	*H. haemolyticus* (2.157)	*H. influenzae* (2.136)	*H. influenzae*
375	Y	N	P	ND	*H. influenzae*	*H. influenzae* (2.376)	*H. haemolyticus* (2.236)	*H. influenzae*

[a]ID, identity; Y, yes; P, positive; N, negative; ND, not determined.
[b]The identification was further confirmed by 16S rRNA gene sequencing.

peaks had area under receiver operating characteristic curve (AUC) values of 0.959 to 1.000 (Table 2). This model could successfully classify all 20 *H. influenzae* and 20 *H. haemolyticus* strains in the validation panel. The other 10 *H. parainfluenzae* and 10 *H. parahaemolyticus* strains were classified as indeterminate, which was defined as neither *H. influenzae* nor *H. haemolyticus*. For clinical isolates, this model correctly classified all 408 *Haemophilus* isolates, including 358 *H. influenzae* and 50 *H. haemolyticus* isolates, to the species level. The ClinProTools mass spectrum model analysis significantly improved the successful identification rate, in comparison to the stand-alone MALDI Biotyper, from 88.2% to 100% (McNemar's test, $P < 0.0001$).

TABLE 2 ClinProTools mass peak statistics for the 7 peaks included in the SVM model[a]

Index	Mass (*m/z*)	DAve	PTTA	PWKW	PAD	Ave1	Ave2	SD1	SD2	CV1	CV2	AUC
1	4,459.73	8.99	<0.000001	<0.000001	<0.000001	0.72	9.71	0.17	5.9	23.59	60.81	0.994
2	4,590.36	2.53	<0.000001	0	<0.000001	3.42	0.89	1.76	0.36	51.49	40.93	0.988
3	4,711.49	16.19	<0.000001	0	<0.000001	22.58	6.4	8.15	5.22	36.07	81.6	0.959
4	4,746.97	11.37	<0.000001	<0.000001	<0.000001	5.15	16.52	3.36	3.44	65.3	20.81	0.987
5	8,918.2	17.31	<0.000001	<0.000001	<0.000001	1.29	18.59	0.2	9.76	15.32	52.51	1.000
6	9,179.4	2.76	<0.000001	0	<0.000001	3.59	0.83	1.46	0.43	40.53	51.16	0.989
7	9,421.92	27.74	<0.000001	0	<0.000001	39.56	11.82	11.58	6.4	29.28	54.09	0.991

[a]DAve, difference between the maximal and the minimal average peak area/intensity of all classes; PTTA, *P* value of *t* test; PWKW, *P* value of Wilcoxon test; PAD, *P* value of Anderson-Darling test; Avg1 and Avg2, peak area/intensity averages of class 1 (*H. influenzae*) and class 2 (*H. haemolyticus*), respectively; SD1 and SD2, standard deviations of the peak area/intensity average of class 1 (*H. influenzae*) and class 2 (*H. haemolyticus*), respectively; CV1 and CV2, coefficients of variation (percent) of class 1 (*H. influenzae*) and class 2 (*H. haemolyticus*), respectively; AUC, area under receiver operating characteristic curve.

DISCUSSION

Discrimination of closely related *Haemophilus* species is of diagnostic importance because of their differences in pathogenicity. Morphological differentiation and biochemical tests are still the standard method used in many laboratories. In our study, we included 358 *H. influenzae* and 50 *H. haemolyticus* clinical isolates. Among the 50 *H. haemolyticus* isolates confirmed by 16S rRNA gene sequencing, 4 isolates had no demonstrable hemolytic activity. This highlighted the presence of nonhemolytic *H. haemolyticus* in our locality. Our data are consistent with the findings from previous studies in Denmark, indicating that the lack of hemolysis should not be used as the critical factor to differentiate *H. haemolyticus* from *H. influenzae* (6, 7). Although the molecular method by specific gene PCR was proven to be superior to biochemical identification (9–11, 20), the long processing time and high running cost made this difficult to be used in routine clinical diagnostic services.

In this study, we proved the concept of using a mass peak analysis software to help laboratory users lacking expertise in mass spectrometry to differentiate closely related bacterial species. We compared the performance of IVD MALDI Biotyper and ClinProTools peak analysis with that of stand-alone MALDI-TOF MS in the differentiation of *H. influenzae* and *H. haemolyticus*. Despite the fact that the most updated MALDI Biotyper reference library includes 27 *H. influenzae* and 21 *H. haemolyticus* mass spectrum profiles, our study demonstrated that a significant number of clinical isolates in our region, 13.1% (47/358) of *H. influenzae* isolates and 2.0% (1/50) of *H. haemolyticus* isolates, could not be identified to the species level by MALDI Biotyper alone. Our results are similar to those reported in a previous publication from China (18). This indicates that the expansion of reference library provided by the manufacturer cannot resolve the identification problem posed by *H. influenzae* and *H. haemolyticus* isolates.

We have developed an SVM mass spectrum analysis model using the ClinProTools software that delivers a practical solution to this current problem. With respect to the classification model, it has to include both reference and clinical strains with confirmed identities. The mass spectra should be prepared by using the ethanol-formic acid extraction method, in order to minimize the noise interference in the mass spectra. The 7 most critical species-specific mass peaks were manually selected into the model mainly based on the AUC values of the peaks. The mass peaks with *m/z* at 4,590.36 Da, 4,711.49 Da, 9,179.40 Da, and 9,421.92 Da were found to be *H. influenzae* specific, while the mass peaks with *m/z* at 4,459.73 Da, 4,746.97 Da, and 8,918.20 Da were *H. haemolyticus* specific (Table 3). Although both the ClinProTools and the IVD MALDI Biotyper analyze the same set of spectral data, the ClinProTools method focusing on the 7 species-specific peaks was demonstrated to have better accuracy than the IVD MALDI Biotyper. By using the ClinProTools SVM model, the rate of successful *H. influenzae* and *H. haemolyticus* identification increased from 88.2% to 100%. This should be closely related to the different analyzing methods used by the two methods.

On the other hand, if only the top-scoring Biotyper identification result was picked and the other identifications with scores of ≥2.0 were neglected, 407/408 (99.8%) isolates could also give correct species-level identifications in the study cohort. How-

TABLE 3 Summary of MALDI-TOF MS results of the 408 *Haemophilus* clinical isolates

Reference identification (biochemical and molecular method)	Species-level identification, no. (%)	
	MALDI Biotyper alone[a]	ClinProTools
H. influenzae ($n = 358$)	311 (86.9)	358 (100)
H. haemolyticus ($n = 50$)	49 (98.0)	50 (100)

[a]Another 48 isolates contained both *H. influenzae* and *H. haemolyticus* in their top 10 identification matches in Bruker MALDI Biotyper.

ever, this "picking rule" has not been statistically evaluated, and thus, there may be a potential risk of misidentification.

The working procedure for the ClinProTools analysis was simple and user-friendly. The hands-on time from importing the spectra file into the ClinProTools software, data analysis, and report generation was approximately 5 min. The recommended workflow would be an initial identification using the IVD MALDI Biotyper system. Isolates with identification of *H. influenzae* or *H. haemolyticus* should be further analyzed by the ClinProTools model analysis for species confirmation.

This study has a few limitations. Since this mass spectrum analysis model is specifically designed for *H. influenzae* and *H. haemolyticus* differentiation, it is not suitable for analyzing species other than *H. influenzae* and *H. haemolyticus*. Although the study demonstrated the feasibility of location-specific improvements to MALDI-TOF MS identification, the ClinProTools method may demonstrate different diagnostic performance for *Haemophilus* strains from different geographical regions. Further evaluation will be required. In conclusion, our study demonstrated the use of the ClinProTools mass spectrum model analysis, which is a reliable, simple, and cost-effective clinical diagnostic method for the differentiation of *H. influenzae* and *H. haemolyticus*. This approach would also be useful for the differentiation of other closely related bacterial species.

MATERIALS AND METHODS

Specimen collection. A total of 408 *Haemophilus* isolates, including 358 *H. influenzae* and 50 *H. haemolyticus* isolates from 363 patients hospitalized between March 2016 and December 2016, were selected for the study. These isolates originated from various clinical specimen types, including 366 sputum specimens, 12 bronchoalveolar lavage specimens, 13 tracheal or bronchial aspirates, and 17 miscellaneous swabs collected from the ear, eye, or anterior nares. The specimens were cultured on Columbia horse blood agar (CHBA) and chocolate agar, incubated at 37°C with 5% CO_2 for 48 h. Isolated Gram-negative rod colonies with small, smooth, and translucent colony morphologies suggestive of *Haemophilus* species were subjected to species identification through biochemical tests, molecular tests, MALDI-TOF MS, and ClinProTools mass spectrum model analysis (21).

Reference biochemical and molecular identification method. Isolated colonies were identified by colony morphology, Gram staining, and basic biochemical tests, including oxidase test, sugar fermentation, and hemin (X factor) and NAD (V factor) dependence. Beta-hemolytic activity was also evaluated by culturing strains on 5% CHBA. *Haemophilus* strains demonstrating beta-hemolysis within 48 h of incubation are considered beta-hemolytic, but hemolytic effect after 48 h is regarded as nonhemolytic.

For identity confirmation, two *H. influenzae*-specific genes, *hpd* and *fucK*, were used in a sequential manner. First, the presence of *hpd* was examined in all 408 isolates by PCR with primers hpd751F (5′-AAAGACCCAAAGGGTTATTGGGTA-3′) and hpd965R (5′-TTACGCACGGTGTAAGGATGCAC-3′). For *hpd*-negative isolates, the presence of *fucK* was further examined by fucK-up (5′-ACCACTTTCGGCGTGG ATGG-3′) and fucK-dn (5′-AAGATTTCCCAGGTGCCAGA-3′) (http://www.mlst.net). Isolates that were *hpd* or *fucK* positive and X and V factor dependent were considered to be *H. influenzae*. On the other hand, *hpd* and *fucK* PCR-negative, X and V factor-dependent isolates would be considered *H. haemolyticus* (9). For isolates with ambiguous PCR results, partial 16S rRNA gene sequencing was performed (8, 22).

Species identification by MALDI Biotyper IVD 2.3. All 408 clinical strains were prepared for MALDI-TOF MS using the manufacturer's recommended direct transfer protocol. A single isolated colony of each strain was inoculated directly onto the MSP96 target plate spot. Each spot was overlaid with α-cyano-4-hydroxy-cinnamic acid matrix (Sigma-Aldrich, St. Louis, MO). The target plate was analyzed by the Bruker microflex LT system (Bruker Daltonics, Bremen, Germany). The protein profile of each spot with *m/z* values of 3,000 to 15,000 was analyzed by the IVD MALDI Biotyper version 2.3 with the spectrum library, V.5.0.0.0 (5,989 spectra). The top 10 identification matches were generated along with confidence scores, ranging from 0.0 to 3.0. According to the manufacturer's CE-IVD certified recommendations, a score of >2.0 indicates promising species-level identification. If two or more different species were shown within the top 10 matches, the species of the isolate would be considered indeterminate.

Species differentiation by ClinProTools. Bruker ClinProTools version 3.0 software was used to develop a classification model for rapid differentiation of *H. influenzae* and *H. haemolyticus*, with

modification from a previous protocol (14). Ten well-characterized *H. influenzae* (2 ATCC standard strains and 8 well-characterized clinical strains) and 10 *H. haemolyticus* clinical reference strains were selected for the construction of a classification model. For each strain, 24 high-quality spectra were prepared using the ethanol formic acid extraction method and were captured using flexControl version 3.4 software (Bruker Daltonics, Bremen, Germany). A mass spectrum model consisting of 480 mass spectra from the 20 *H. influenzae* or *H. haemolyticus* reference strains was created in the ClinProTools. Spectrum pretreatment and peak calculation operations were performed using the preset configuration. A classification model was generated using the support vector machine (SVM) algorithm. The number of peaks selected in the model was limited to 10, and only peaks with area under receiver operating characteristic curve (AUC) values of >0.9 were considered (23). The recognition capability (RC) and cross validation (CV) percentages of the SVM model were generated to demonstrate the reliability and accuracy of the model. RC and CV percentages were indicators of the model's performance and were useful predictors of the model's ability to classify test isolates. The model was then validated blindly by 3 laboratory technicians with archived clinical strains: 20 *H. influenzae* isolates, 20 *H. haemolyticus* isolates, and 20 other *Haemophilus* isolates (10 *H. parainfluenzae* and 10 *H. parahaemolyticus* isolates). The model showing 100% accuracy was selected for further testing. For the 408 clinical strains, the corresponding spectra created by the direct transfer method during the IVD MALDI Biotyper identification were imported into ClinProTools and were further analyzed with the SVM classification model for *H. influenzae* and *H. haemolyticus* differentiation.

Statistical analysis. The identification accuracies of IVD MALDI Biotyper alone and IVD MALDI Biotyper plus ClinProTools analysis were compared using chi-square test and McNemar's test in MedCalc software version 14.12.0 (MedCalc Software, Ostend, Belgium).

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We declare no conflict of interest.

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