Identification of Neisseria meningitidis by MALDI-TOF MS may not be reliable.

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The identification of *Neisseria meningitidis* by MALDI-TOF may not be reliable.

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Running title: Identification of meningococci by MALDI-TOF

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Abstract

Objectives: The Matrix Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF/MS) technique is increasingly used in hospital laboratories for routine identification of microorganisms. However, its performance is variable particularly for highly variable species such as *Neisseria meningitidis*. Reliable identification of *N. meningitidis* is crucial for the management of invasive meningococcal disease by rapid implementation of treatment and preventive measures among close contacts. We aim here in this study to assess and improve *N. meningitidis* identification by MALDI-TOF by enriching the databases with reference strains identified using whole genome sequencing (WGS) as a gold standard.

Methods: We first built-up a collection of 24 strains from several species of *Neisseria* genus that we characterized by WGS. This collection was added to the available database to test by MALDI-TOF/MS another collection of 32 clinical isolates received between 2015 and 2017 at the French National Reference Laboratory for Meningococci.

Results: Using the commercially available library of mass spectrometry profiles (MSP), only 67% (47-82%, 95% CI) of concordance was observed at the species level between MALDI-TOF and WGS characterization. However, when the new enriched reference collection was used on the second subset of isolates, the identification of *N. meningitidis* was significantly improved (p=0.0016) showing 92% (75-98%, 95% CI) of specificity while it was of 52% (34-70%, 95% CI) with the manufacturer's database alone.

Conclusions: Our data highlight the need to update the available MALDI-TOF database by high quality references to enhance the identification of *N. meningitidis* and avoid unwarranted preventive measures or missing them.

Keywords:

MALDI-TOF, whole genome sequencing, *Neisseria meningitidis*, identification

Introduction

The Matrix Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF/MS) technique is increasingly used in hospital clinical laboratories as routine tool for microbial identification. It is based on generating a unique protein mass spectrum of a given microorganism and comparing it to a library of spectra from known reference microorganisms (1-3). MALDI-TOF/MS is a simple, rapid and cost-effective identification technique(4). It is also time saving by identifying organism in positive blood culture broths or urine samples (5-7).

Bacterial identification is quantified by the score obtained by comparison to a library of main spectra profiles (MSP) present in the database. This score reflects the level of reliability of the identification at the genus or species levels. However, the reliability of the identification depends on the representativeness of the reference strains included in the MSP library. This issue is critical for genus and species showing high diversity and frequent horizontal DNA exchanges such as the genus *Neisseria* (8). A global gene pool has been suggested to be shared by the species of this genus including the pathogenic species *Neisseria meningitidis* and *Neisseria gonorrhoeae*(9). Indeed, MALDI-TOF/MS as well as the conventional phenotypic and biochemical testing for the characterization of *Neisseria* species can lead to misidentifications that may be also due to poor representativeness of available MSPs from *Neisseria* species other than pathogenic *Neisseria* species (10-12). The high degree of genetic relatedness between *N. meningitis*, *Neisseria polysaccharea*, *N. gonorrhoeae*, *Neisseria lactamica* and *Neisseria cinerea* species promoted the suggestion that these species form a

single genospecies on the basis of the stability of DNA heteroduplex to the S1 nuclease activity (13). However, next generation sequencing era and the analysis of whole genome sequences (WGS) data showed that species of the genus *Neisseria* could be robustly identified by a set of 53 genes encoding ribosomal protein subunits (ribosomal multilocus sequence typing, rMLST). The rMLST scheme represents an effective and rapid method for taxonomic classification (14). We aimed in this work to assess the use of MALDI-TOF/MS in the identification of *Neisseria* species and to improve the specificity of *N. meningitidis* identification by MALDI-TOF/MS. The analysis was conducted using the WGS data as reference to ascertain the bacterial identification.

Methods.

Strain collections

We used two collections of strains: The first one (reference collection) corresponded to 24 strains from the collection of the French National Reference Laboratory for Meningococci from 1979 to 2004 with at least one strain of each *Neisseria* species present in our collection. These strains were characterized initially by conventional phenotypic and biochemical testing (PubMLST *Neisseria* database: id38974, id38975, id38976, id38977, id38978, id38979, id38980, id38981, id38982, id38983, id38984, id38985, id38986, id39715, id39716, id39717, id39718, id39719, id39720, id39721, id39722, id39723, id39724, id39725).

The second collection (test collection) corresponded to 25 non-*N. meningitidis* strains, isolated between 2015 and 2017 as well as 7 *N. meningitidis* strains representing each of the serogroups A, B, C, W, X, Y in addition to a non-groupable strain (PubMLST *Neisseria* database: id40248, id40261, id40270, id41220, id42055, id42057, id42058, id42059, id42060, id42061, id42062, id42198, id42421, id43987, id45355, id45356, id46114, id49258, id49259, id51008, id51545, id51557, id51558, id51584, id52432, id53422, id57256, id57266, id57277, id58872, id58873, id58874).

Whole genome sequencing and data analysis

Genomic DNA was extracted with the MagNA Pure 96 system (Roche Molecular System, Pleasanton, USA). Libraries preparation were performed with the Nextera® XT DNA library Preparation Kit (Illumina, San Diego, USA) and whole genome sequencing was processed with Illumina technology (NextSeq 500, Illumina) with paired-end strands of 150 bp and with a sequencing depth of 50X.

All de novo assemblies were performed with SPAdes (CAB, St. Petersburg State University, Russia) and genome comparison was performed using the BIGSdb Genome Comparator tool available in PubMLST platform (15). Core genomes were compared with 50 isolates from several species of *Neisseria*, available in PubMLST *Neisseria* database (id5354, id14740, id19080, id19081, id19083, id19087, id19088, id19089, id19090, id19095, id19096, id19097, id19100, id19940, id20515, id20516, id21038, id21039, id21040, id21041, id21042, id21044, id21045, id21046, id21048, id21049, id21061, id21063, id21064, id26870, id26871, id26876, id27632, id28271, id29271, id36140, id36153, id36167, id36173, id36174, id36179, id36317, id37587, id40839, id41201, id41237, id41396, id41409, id41588, id49340). A matrix of allelic distance was computed based on rMLST scheme and viewed in SplitsTree4 (version 4.14.6) using the Neighbor-Net algorithm (16).

MALDI-TOF/MS analysis

Ribosomal proteins extractions were performed according to the manufacturer's instructions with a formic acid and acetonitrile treatment. Proteins were spotted in duplicate on the MALDI biotarget and co-crystallized with HCCA matrix. Spectra were acquired with a MALDI Biotyper Microflex LT (Bruker Daltonik GmbH, Bremen, Germany).

In order to build a local database, the strains selected (reference collection) were spotted 8 times in the same target and then read 4 times, generating 32 spectra for each strain. A minimum of 20 spectra for each strain were selected after quality control procedures, in order to create a single Main Spectra Profile (MSP) for each strain of our database (FlexAnalysis software, version 3.4).

Strain identification was evaluated by the score generated from the Bruker Biotyper software (MBT Compass and Explorer, version 4.1.80) against all biotypes available in the Bruker's database (7311 MSP available).

A score comprised between 2.0 to 3.0 leads to a reliable identification at the genus and species level. If the score is comprised between 1.7 and 2.0, the identification of the genus remains probable and finally if the score is under 1.7, the reliability is not sufficient to let a genus identification.

Specificity and sensitivity were calculated using a two-way table as previously described (17) with the genomic identification as a gold standard. We used Chi^2-Mc Nemar test to calculate the significance of the differences among the specificities of the tests used to identify bacterial isolates (WGS and MALDI-TOF/MS).

Results

WGS characterization of the collection

We have selected several strains (N=24) from several species of the genus *Neisseria* that mainly were not or poorly represented in the available database of MSP in the Bruker MALDI-TOF/MS system (Table 1). The identification of theses strains was initially performed using the conventional biochemical identification of *Neisseria* (18) as indicated in Materials and Methods. We next used WGS on all these strains and extract the rMLST that analyses the genetic relationships on the basis of the polymorphism of 53 *rps* genes encoding ribosomal proteins. This analysis allowed identification of species by comparison to rMLST profiles already available on the BIGSdb platform as previously described (19). This analysis compared the strains of our collection to the 50 strains belonging to different species of the genus *Neisseria* that were available in the BIGSdb database. The neighbor-joining tree using rMLST data showed species-specific clusters as previously described (Figure A) (20). This genetic identification was consistent with the initial biochemical identification for only 17/24 of our collection of strains (71%). However, for 7 isolates, no consistent identification was confirmed comparing genetic to phenotypic identification (isolates highlighted with blue circles, Figure A). One strain (id38974) initially identified as *N. perflava* appeared to belong to the *N. mucosa* cluster by rMLST and thus further underlines the difficulty in the identification of *Neisseria* species by the conventional biochemical tests. Three other strains (id38979; id39722 and id39723) that were biochemically identified respectively as *N. flava*, *N. sicca* and *N. perflava*, clustered together in the *N. subflava* group by rMLST (*N. subflava biovars*)(14). The sequence analysis of *rplF* gene (encoding the 50S ribosomal protein L6) also assigned all of these three isolates as *N. subflava* (20). One isolate that was identified as *N. polysaccharea* (id39718) was characterized by *rplF* sequence as *N. bergeri*, a closely

190 species related to *N. polysaccharea* (14). And finally, the two isolates (id39719 and id39720)
191 identified as *Neisseria denitrificans* and *Neisseria canis* clustered together on the rMLST-
192 based tree but were separated from the 50 isolates in the BIGSdb database (Figure A).
193 In order to refine the rMLST identification of our set of 24 strains, particularly for the three
194 last strains mentioned above, a core genome MLST (cgMLST) analysis was performed
195 (Figure B), that analyses the genetic relationships on the basis of polymorphism of the core
196 genome (1605 genes cgMLST) (15). This analysis confirmed the data obtained with the
197 rMLST analysis. In addition, the strain (id39718) that was not identified by rMLST (but was
198 identified as *N. bergeri* by *rplF* sequencing) clustered by cgMLST with the *N. polysaccharea*
199 group (Figure B). Finally, the two strains that were identified initially by biochemical tests as
200 *N. denitrificans* and *N. canis* (but were not identified by rMLST) clustered with the *Neisseria*
201 species and are more closely related to commensal species isolated from animal hosts. These
202 two isolates were assigned as *Neisseria sp.*, in absence of reference strains available in the
203 database (Table 1).
204
205 **MALDI-TOF/MS characterization**
206 We next performed the MALDI-TOF analysis to identify the 24 isolates using the Microflex
207 Biotyper Bruker system that allowed the identification at genus level for 96% of our set of
208 strains (23/24 strains with a score above 1.7). Among these 23 isolates, 19 isolates reached a
209 score higher than 2, allowing a species level identification for 79 % (19/24) of our reference
210 collection. The remaining strain showed a score under 1.7 that do not permit species
211 identification (Table 1).
212 The MALDI-TOF identification of the 24 strains was consistent with the genomic analysis for
213 16/24 strains (67% of total strains) at species level (Table 1). Indeed, most of isolates (5/8

isolates) that unmatched between MALDI-TOF and genomic data, showed MALDI-TOF scores higher than 2 (Table 1). Most striking is that 5 isolates were identified by MALDI-TOF as *N. meningitidis* with scores ranging between 1.65 and 2.35 but were identified by rMLST and cgMLST as *N. polyscharrea*, *N. bergeri* or *N. cinerea* (Table 1). The *N. meningitidis* strain that was included within the 24 strains (id38983) was correctly identified with a score of 2.58. These data indicate low specificity (78%) for *N. meningitidis* identification by MALDI-TOF, although it showed high sensitivity (100%).

Improvement of the MALDI-TOF diagnosis of *N. meningitidis*

The 32 isolates from the test collection (see Methods) were sequenced and were further identified by rMLST and cgMLST (Figure A, Figure B and Table 2). All these strains (the 25 non-*N. meningitidis* strains and the 7 *N. meningitidis* strains) were subsequently tested by MALDI-TOF and identified using both databases (the original database and the extended one as described in Methods section). All the 7 *N. meningitidis* strains were correctly identified by both databases. For the 25 "non-*N. meningitidis*" isolates and using the original Bruker database, the identification reached the genus level for all tested strains and 20/25 (80%) at species level (Table 2). However, 12/25 (48%) strains were misidentified as *N. meningitidis* (indicated with red stars in Figure A) leading to a specificity of *N. meningitidis* identification of 52% (34-70%, 95% CI). When the extended database was used for the identification of spectra, only two of the 12 false positive *N. meningitidis* were still identified as *N. meningitidis* (id40248 and id45356) with scores >2. Both strains were identified as *N. polysaccharea* by cgMLST (Table 2). However, two strains (id42059 and id43987) were identified as *N. bergeri* with the extended database (scores of 1.89 and 2.17 respectively) whereas they were characterized as *N. cinerea* and *N. polysaccharea* on the basis of genomic

database respectively. Using these results, the extended database allowed an identification of *N. meningitidis* by MALDI-TOF within these 32 strains with a specificity of 92% (75-98%, 95% CI) and a sensitivity of 100% (65-100%, 95% CI).

Discussion

In this work we described a new MALDI-TOF database that is based on WGS as a gold standard to identify bacterial species. Indeed, our data suggest that identification may be problematic for bacterial species such as those of the genus *Neisseria* where free and frequent horizontal DNA exchanges may occur. Indeed, the data clearly showed that the currently available database showed low specificity for the identification of *N. meningitidis* resulting in a high level of false positive diagnosis of this species. Concordantly, some strains tested in our study that were misidentified as *N. meningitidis* by MALDI-TOF/MS (*N. cinerea* and *N. polysaccharea*) were from invasive infections and were isolated from blood (Table 1 and 2). The performance of MALDI-TOF can be improved by enriching the Bruker database with spectra from poorly represented species and from species that are close to *N. meningitidis*. Indeed, most of isolates identified as *N. meningitidis* by mass spectrometry, actually belonged all to *N. cinerea* species and *N. polysaccharea* polyphyletic group. With the expansion of Bruker MSP by our database (MSP based on genomic data analysis), we were able to improve the diagnosis of *N. meningitidis* with higher specificity (92% versus 52%). Enriching the database of the MALDI-TOF Bruker system was also reported to improve the identification of *N. gonorrhoeae* (21). Our work presents the originality of using WGS data as a gold standard to validate the identification of bacterial species. Genomic-based identification may be suggested as a gold standard to build up new collection of reference strains that can be used to construct spectra databases. However our work included a limited number of isolates

belonging to non-*Neisseria meningitidis* species and this may explain that two isolates were still identified as *N. meningitidis* by using the expanded spectral database, whereas these isolates clustered within the *N. polysaccharea* group. This highlights the need to increment and frequently update the MSP database based on genome comparison analysis, in order to enhance the performance of *Neisseria* species identification by mass spectrometry and conducting similar work on other commercially available databases is needed. Our work is highly relevant as bacterial identification by MALDI-TOF/MS is becoming widely used in clinical microbiology laboratories due to its simplicity, rapidity and low cost. Indeed, it provides within few minutes a bacterial identification on microbial colonies or blood cultures. This diagnosis is of concern as *Neisseria meningitidis* can be encountered in invasive bacterial disease leading to unwarranted uses of prophylactic antibiotics. Our new database is expected to improve the quality of this rapid diagnosis and should hence be beneficial for rapid management of meningococcal disease.

Transparency Declaration

All authors report no conflicts of interest relevant to this article.

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Foundation Meningococcus Genome Library (http://www.meningitis.org/research/genome) developed by Public Health England, the Wellcome Trust Sanger Institute and the University of Oxford.

Author contributions

E.H. and M.K.T. conceived and designed the experiments; E.H and Y.B. performed the experiments; E.H. and M.K.T. analysed the data; E.H wrote the manuscript with input from M.K.T. All authors reviewed and edited the manuscript.

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Legends of figures and tables

Figure. Characterization of isolates by genome analysis. Neighbor-joining analysis of rMLST (**A.)** and cgMLST (**B.)** of our two collections (reference and test) (56 isolates) with 50 available isolates in the BIGSdb database. Discordance between genomic diagnosis and phenotypic and biochemical tests are indicated with blue circles or branches (N=7). Concordant identification between genomic data analysis and MALDI-TOF identification with Bruker's database are indicated with green branches. Red branches stand for a discordance between genomic diagnosis and MALDI-TOF identification with Bruker's database. Misidentification of *N. meningitidis* by MALDI-TOF diagnosis with Bruker's database are indicated with red stars.

Fig.A

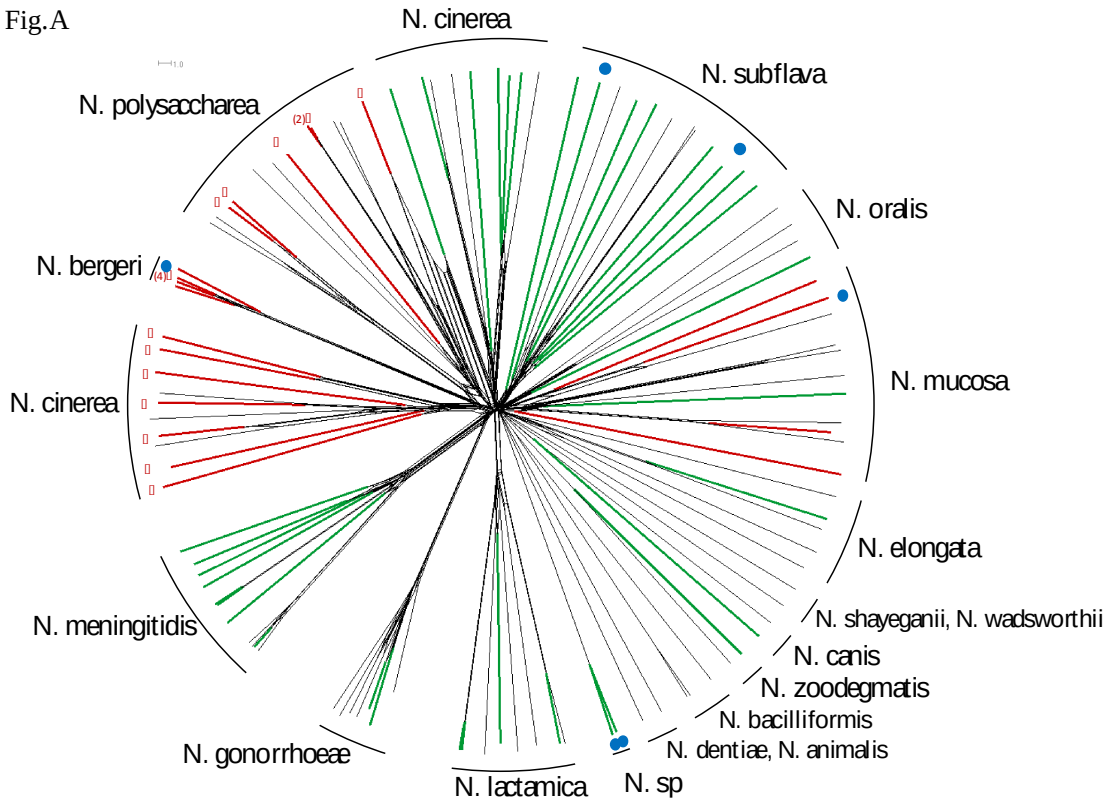

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Fig.B

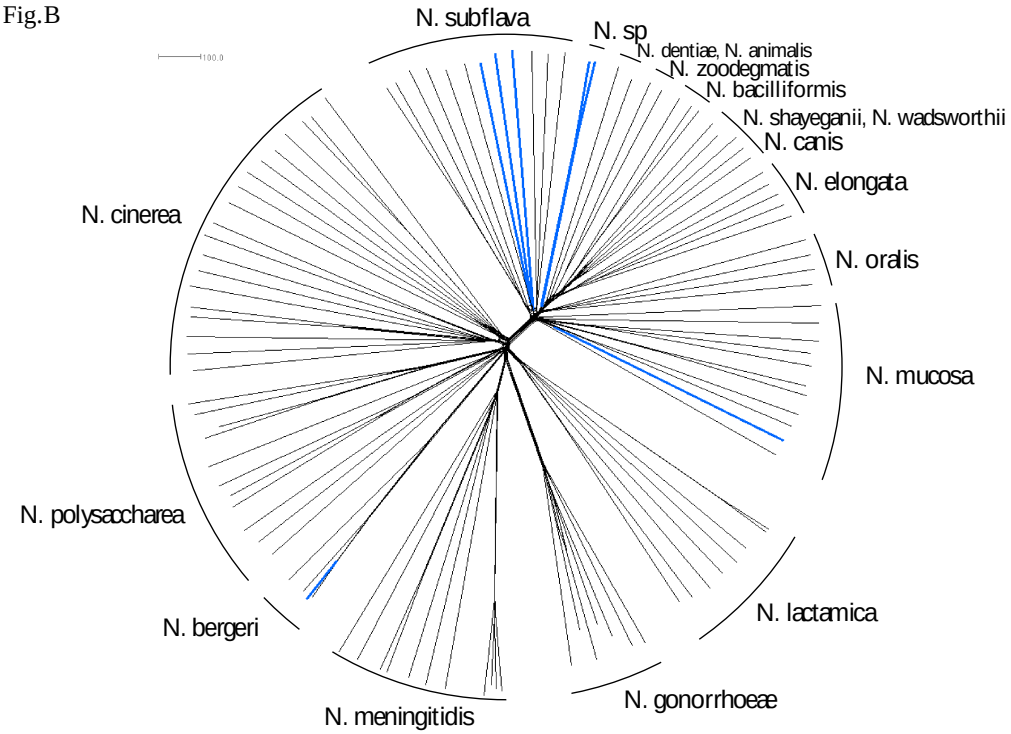

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Table 1. Characterization of our collection of reference strains by biochemical tests, rMLST analysis, cgMLST analysis and MALDI-TOF. *N. meningitidis* misidentifications by MALDI-TOF are highlighted in grey. PubMLST id (i) indicates the strain was isolated from an invasive site.

PubMLST id	Biochemical identification	rMLST identification	cgMLST identification	BRUKER MALDI-TOF identification	BRUKER MALDI-TOF mean score
38974 (i)	N. perflava	N. mucosa	N. mucosa	N. macacae	2,13
38975 (i)	N. lactamica	N. lactamica	N. lactamica	N. lactamica	2,51
38976	N. subflava	N. subflava	N. subflava	N. subflava	2,41
38977	N. mucosa	N. mucosa	N. mucosa	N. macacae	2,15
38978	N. cinerea	N. cinerea	N. cinerea	N. cinerea	2,48
38979	N. flava	N. subflava	N. subflava	N. subflava	2,57
38980 (i)	N. polysaccharea	N. polysaccharea	N. polysaccharea	N. meningitidis	2,31
38981	N. polysaccharea	N. polysaccharea	N. polysaccharea	N. meningitidis	2,34
38982	N. gonorrhoeae	N. gonorrhoeae	N. gonorrhoeae	N. gonorrhoeae	2,53
38983 (i)	N. meningitidis	N. meningitidis	N. meningitidis	N. meningitidis	2,58
38984	N. cinerea	N. cinerea	N. cinerea	N. cinerea	2,53
38985 (i)	N. sp. close to N. lactamica	N. lactamica	N. lactamica	N. lactamica	2,57
38986 (i)	N. cinerea	N. cinerea	N. cinerea	N. meningitidis	1,65
39715	N. mucosa	N. mucosa	N. mucosa	N. subflava	1,89
39716	N. canis	N. canis	N. canis	N. canis	2,23
39717 (i)	N. cinerea	N. cinerea	N. cinerea	N. cinerea	2,22
39718	N. polysaccharea	N. bergeri	N. bergeri	N. meningitidis	2,35
39719	N. denitrificans	N. sp.	N. sp.	Bergeriella denitrificans	1,76
39720	N. canis	N. sp.	N. sp.	Bergeriella denitrificans	1,85
39721	N. cinerea		N. cinerea	N. cinerea	2,35
39722 (i)	N. sicca	N. subflava	N. subflava	N. subflava	2,43
39723 (i)	N. perflava	N. subflava	N. subflava	N. subflava	2,52
39724	N. cinerea	N. cinerea	N. cinerea	N. meningitidis	1,84
39725	N. sp. close to N. zoodegmatis	N. zoodegmatis	N. zoodegmatis	N. zoodegmatis	2,59

Table 2. Improvement of MALDI-TOF identification against a test collection of strains by implementing profiles (reference collection) to the manufacturer's database. *N. meningitidis* misidentifications by MALDITOF are highlighted in grey. PubMLST id (i) indicates the strain was isolated from an invasive site.

PubMLST id	Biochemical identification	rMLST identification	cgMLST identification	BRUKER MALDI-TOF identification	BRUKER MALDI-TOF mean score	BRUKER+MSP MALDI-TOF identification	BRUKER+MSP MALDI-TOF mean score
40248	N. sp.	N. polysaccharea	N. polysaccharea	N. meningitidis	2,29	N. meningitidis	2,29
40261 (i)	N. sicca	N. subflava	N. subflava	N. subflava	2,16	N. subflava	2,32
40270 (i)	N. meningitidis	N. subflava	N. subflava	N. subflava	2,28	N. subflava	2,43
41220	N. polysaccharea	N. bergeri	N. bergeri	N. meningitidis	2,21	N. bergeri	2,61
42055 (i)	N. mucosa	N. oralis	N. oralis	N. oralis	2,34	N. oralis	2,34
42057	N. cinerea	N. cinerea	N. cinerea	N. meningitidis	1,97	N. cinerea	2,19
42058	N. cinerea	N. cinerea	N. cinerea	N. meningitidis	1,97	N. cinerea	2,22
42059	N. cinerea	N. cinerea	N. cinerea	N. meningitidis	1,91	N. bergeri	1,89
42060	N. sicca	N. subflava	N. subflava	N. subflava	2,26	N. subflava	2,29
42061	N. sp.	N. cinerea	N. cinerea	N. cinerea	2,24	N. cinerea	2,28
42062	N. sp.	N. cinerea	N. cinerea	N. meningitidis	1,92	N. cinerea	2,13
42198	N. lactamica	N. lactamica	N. lactamica	N. lactamica	2,39	N. lactamica	2,43
42421 (i)	N. cinerea	N. cinerea	N. cinerea	N. meningitidis	1,86	N. cinerea	2,24
43987	N. polysaccharea	N. polysaccharea	N. polysaccharea	N. meningitidis	2,22	N. bergeri	2,17
45355	N. mucosa	N. mucosa	N. mucosa	N. macacae	2,06	N. mucosa	2,55
45356	N. polysaccharea	N. polysaccharea	N. polysaccharea	N. meningitidis	2,22	N. meningitidis	2,22
46114 (i)	N. mucosa	N. mucosa	N. mucosa	N. mucosa	2,25	N. mucosa	2,45
49258	N. cinerea	N. cinerea	N. cinerea	N. cinerea	2,39	N. cinerea	2,40
49259	N. polysaccharea	N. bergeri	N. bergeri	N. meningitidis	2,22	N. bergeri	2,55
51008 (i)	N. meningitidis	N. meningitidis	N. meningitidis	N. meningitidis	2,47	N. meningitidis	2,47
51545	N. gonorrhoeae	N. gonorrhoeae	N. gonorrhoeae	N. gonorrhoeae	2,35	N. gonorrhoeae	2,35
51557 (i)	N. meningitidis	N. meningitidis	N. meningitidis	N. meningitidis	2,48	N. meningitidis	2,48
51558 (i)	N. meningitidis	N. meningitidis	N. meningitidis	N. meningitidis	2,57	N. meningitidis	2,57
51584	N. meningitidis	N. meningitidis	N. meningitidis	N. meningitidis	2,47	N. meningitidis	2,47
52432	N. sicca	N. subflava	N. subflava	N. subflava	2,32	N. subflava	2,08
53422 (i)	N. meningitidis	N. meningitidis	N. meningitidis	N. meningitidis	2,52	N. meningitidis	2,52
57256 (i)	N. elongata	N. elongata	N. elongata	N. elongata	2,51	N. elongata	2,51
57266 (i)	N. meningitidis	N. meningitidis	N. meningitidis	N. meningitidis	2,56	N. meningitidis	2,56
57277 (i)	N. meningitidis	N. meningitidis	N. meningitidis	N. meningitidis	2,53	N. meningitidis	2,53
58872	N. lactamica	N. lactamica	N. lactamica	N. lactamica	2,37	N. lactamica	2,37
58873	N. polysaccharea	N. polysaccharea	N. polysaccharea	N. meningitidis	2,36	N. polysaccharea	2,33
58874	N. polysaccharea	N. bergeri	N. bergeri	N. meningitidis	2,13	N. bergeri	2,24