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Direct Bacterial Profiling by Matrix-Assisted Laser Desorption–Ionization Time-of-Flight Mass Spectrometry for Identification of Pathogenic *Neisseria*

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The present study investigates the suitability of direct bacterial profiling as a tool for the identification and subtyping of pathogenic *Neisseria*. The genus *Neisseria* includes two human pathogens, *Neisseria meningitidis* and *Neisseria gonorrhoeae*, as well as several nonpathogenic *Neisseria* species. Here, a matrix-assisted laser desorption/ionization time-of-flight mass spectrometry profiling protocol was optimized using a laboratory strain of *E. coli* DH5α to guarantee high quality and reproducible results. Subsequently, mass spectra for both laboratory and clinical strains of *N. gonorrhoeae*, *N. meningitidis*, and several nonpathogenic *Neisseria* species were collected. Significant interspecies differences but little intraspecies diversity were revealed by means of a visual inspection and bioinformatics examination using the MALDI BioTyper software. Cluster analysis successfully separated mass spectra collected from three groups that corresponded to *N. gonorrhoeae*, *N. meningitidis*, and nonpathogenic *Neisseria* isolates. Requiring only one bacterial colony for testing and using a fast and easy measuring protocol, this approach represents a powerful tool for the rapid identification of pathogenic *Neisseria* and can be adopted for other microorganisms. *(J Mol Diagn 2009, 11:75–86; DOI: 10.2353/jmoldx.2009.080079)*

The prevalence of related diseases makes accurate identification of pathogenic *Neisseria* difficult. Gonococcal infection, caused by *Neisseria gonorrhoeae*, is still one of the most common bacterial sexually transmitted diseases in the world, estimated to infect more than 60 million people each year. The incidence of gonorrhea is lowest in Western Europe, but the disease remains epidemic in Eastern Europe, much of Africa, the Indian subcontinent, and parts of Asia, South America, and the United States. In Russia, more than 89,000 cases of gonorrhea were diagnosed in 2005.[1] *Neisseria meningitidis* is a common inhabitant of the mucosal membranes of the nose and throat; infections occur sporadically throughout the world with seasonal variations and account for a proportion of endemic bacterial meningitis. Although the highest burden of disease currently exists in Africa, epidemics can occur in any part of the world. Asia has suffered from some major epidemics of meningococcal disease in the last 30 years (China 1979 and 1980, Vietnam 1977, Mongolia 1973–1974 and 1994–1995, Saudi Arabia 1987, Yemen 1988). There have also been epidemics in Europe and in America during the last 30 years, but they have not reached the very high incidence levels of epidemics in other parts of the world. In Russia, the frequency of bacterial meningitis and sepsis caused by *N. meningitidis* reaches 5000 per year with mortality rates from 5% to 20%.[2]

Conventional diagnostic methods for *Neisseria* species identification include series of enzyme reaction tests, which have a certain number of false negative results.[3] As taxonomic differences between members of the *Neisseria* genus are small, the identification of these pathogens remains problematic and as such, no single method is currently recommended for a definitive identification. Prolyliminopeptidase was previously considered to be almost universally present in *N. gonorrhoeae*, but the widespread occurrence of Prolyliminopeptidase-negative *N. gonorrhoeae* strains[4] potentially results in incorrect, doubtful, or delayed species identification. Additionally, *N. meningitidis* strains producing Prolyliminopeptidase like *N. gonorrhoeae* have been reported.

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Traditional microbiological methods based on morphological and biochemical characteristics are time-consuming, error-prone, and laborious. Therefore, the development of new rapid and effective methods for *Neisseria* species identification is required. Mass spectrometry is a powerful routine tool in biological studies and presents an attractive alternative to traditional methods, in particular for bacterial identification and differentiation.

Table 1. Bacterial Strains

Strain no.	Description		Isolation region/additional information
E. coli			
1	Ec DH5alpha		Laboratory strain DH5α
Neisseria gonorrhoeae			
	Por type		
1	Ng ATCC 49226		Reference strain ATCC 49226
2	Ng a04	PIB 26	Archangelsk
3	Ng a032	PIA 6	Archangelsk
4	Ng a040	PIA 6	Archangelsk
5	Ng a041	PIB 3	Archangelsk
6	Ng ch012	PIB 1	Cheboksary
7	Ng e004	PIB 2	Yekaterinburg
8	Ng i6001	PIB 2	Irkutsk
9	Ng i6002	PIB3	Irkutsk
10	Ng i6003	PIB 22	Irkutsk
11	Ng i6004	PIB22	Irkutsk
12	Ng i6005	PIA 2	Irkutsk
13	Ng i6007	PIB 3	Irkutsk
14	Ng i6008	PIA 6	Irkutsk
15	Ng i6010	PIB 3	Irkutsk
16	Ng i6011	PIB 22	Irkutsk
17	Ng i6012	PIB 22	Irkutsk
18	Ng i6013	PIA 10	Irkutsk
19	Ng ka003	PIB 2	Kaluga
20	Ng ka005	PIB 2	Kaluga
21	Ng m024	PIB 2	Murmansk
22	Ng mr012	PIB 3/6	Moscow region
23	Ng n012	PIB3	Nizhniy Novgorod
24	Ng n020	PIB 2	Nizhniy Novgorod
25	Ng ps601	PIB 3	Pskov
26	Ng sp030	PIB 8	Saint-Petersburg
27	Ng sp031	PIB 3/6	Saint-Petersburg
28	Ng sp032	PIB 2	Saint-Petersburg
29	Ng sp033	PIB 2	Saint-Petersburg
30	Ng sp034	PIB 3/6	Saint-Petersburg
Neisseria meningitidis			
	Serogroup		
1	Nm 229	A	Moscow region
2	Nm 537	B	Moscow region
3	Nm 549	A	Moscow region
4	Nm 552	A	Moscow region
5	Nm 592	A	Moscow region
6	Nm 605	A	Moscow region
7	Nm 266	A	Moscow region
8	Nm 441	B	Moscow region
9	Nm 406	A	Moscow region
10	Nm 634	B	Moscow region
11	Nm 639	A	Moscow region
12	Nm 1522	B	Moscow region
13	Nm 1539	A	Moscow region
14	Nm 1541	C	Moscow region
15	Nm 070309	A	Moscow region
Nonpathogenic *Neisseria*			
1	*N. elongata* 605		Moscow region
2	*N. flavescens* 533		Moscow region
3	*N. flavescens* 8552		Moscow region
4	*N. lactamica* 79		Moscow region
5	*N. mucosa* 839		Moscow region
6	*N. mucosa* 1591		Moscow region
7	*N. perflava* 1621		Moscow region
8	*N. polysaccharea* 547		Moscow region
9	*N. sicca* 70		Moscow region
10	*N. flava* 110		Moscow region
11	*N. subflava* 239		Moscow region
12	*Neisseria* spp 8503		Moscow region
13	*Neisseria* spp 8553		Moscow region
14	*N. subflava* 227		Moscow region
15	*N. subflava* 1672		Moscow region

Direct bacterial profiling (DBP) by means of matrix-assisted laser desorption–ionization time-of-flight (MALDI-TOF) mass spectrometry has been suggested as an alternative approach for rapid and accurate classification and species identification of microbes,[5–9] overcoming the existing limitations of classical biochemical methods. The most important advantages are: 1) requirement of only a small amount of biological material, 2) a fast and easy measurement protocol, and 3) high specificity in species differentiation.

Although the method has been described several years ago there is no common protocol of sample preparation for mass spectrometry measurement. Suggested procedures include "intact" cells technique (native cells are deposited directly onto the sample holder and mixed with matrix),[10] different ways of protein extraction, and even the utilization of non-ionic surfactants to expand the upper mass range for bacterial proteins obtained from whole cell MALDI-TOF mass spectrometry analysis.[11] The influence of cultivation condition, matrix, solvent, and spotting technique on the reproducibility and quality of mass spectra has been reported.[12] Therefore, today the majority of studies in this field are intended to develop effective and universal sample preparation and measurement protocols and the optimization of analytical algorithms. Whether it will be a single protocol for all samples or several techniques for different microbial groups, the future will show. Further studies will have to prove the applicability of mass spectrometry profiling for the reliable identification of clinically relevant microorganisms in routine and diagnostic settings.

Here, we present an evaluation of DBP for the identification of pathogenic *Neisseria* and of its ability to distinguish between closely related species from the genus *Neisseria*. For this purpose, a dedicated database of mass spectra has been created using the recently presented MALDI Biotyper bioinformatics package.[13] Furthermore, the intrageneric and intraspecies variability of DBP mass spectra was analyzed.

Materials and Methods

Bacterial Strains

Clinical strains of *N. gonorrhoeae*, *N. meningitides,* and non-pathogenic *Neisseria* ($n = 29$, $n = 13$, and $n = 15$, respectively) were collected. Additionally, ATCC strain 49226 of *N. gonorrhoeae* was included for validation of assay performance. A laboratory strain of *Escherichia coli* DH5α (Life Technologies, UK) was used for mass spectrometry measurement optimization. Detailed information about the strains is noted in Table 1.

Before analysis, *E. coli* strain DH5α was grown on lysogeny broth (LB) agar plates at 37°C for 18 hours. *N. gonorrhoeae* strains were grown on the medium prepared by supplementing GC agar base (BBL-DIFCO) with 1% of defined supplement (IsoVitaleX; BBL-DIFCO) at 37°C in 5% CO_2 atmosphere for 18 hours. Crystal Haemophilus/*Neisseria* (BBL-DIFCO) test was used for species identification. *N. meningitidis* strains as well as non-pathogenic *Neisseria* were cultured using special media "Chocolate Agar" or "Columbia III" (BBL-DIFCO) with 5% sheep blood at 37°C in 5% CO_2 atmosphere for 19 to 24 hours. Biochemical species identification was performed by the automated identification system WalkAway40 (MicroScan Dade Behring) using Hemophilus and Neissena Identification (HNID) kit-panels. Serotyping of *N. meningitidis* strain was done by agglutination test.

Por *Typing of* N. gonorrhoeae *Isolates*

The *N. gonorrhoeae* typing based on analysis of *por* gene variability was done as described previously.[14–17] The *por*-type of a particular isolate was determined by identification of the obtained nucleotide sequences of the *por* genes in the EMBL (European Molecular Biology Laboratory) and NCBI (National Center for Biotechnology Information) databases.

Bacterial Preparation for MALDI Mass Spectrometry Analysis

For MALDI-TOF mass spectrometry analysis single colonies of fresh bacterial cultures have been used. Cells were transferred from the plate into an extraction 0.5 ml tube (Eppendorf, Germany) with a 1.0 μl plastic loop (FL medical, Italy) corresponding to 5 to 10 mg cell material, approximately. Then, 50 μl of extraction solution consisting of 50% acetonitrile (Sigma-Aldrich, Germany), 2.5% trifluoroacetic acid (Sigma-Aldrich, Germany), and 47.5% water (Fluka, Switzerland) was added. The mixture was resuspended well and centrifuged (12000 rpm for 1 minute). Supernatant was used for further mass spectrometry analysis.

Saturated solutions of α-cyano-4-hydroxy cinnamic acid, sinapinic acid, or ferulic acid (all substances from Bruker Daltonics, Germany) were prepared in 1.0 ml of 50% acetonitrile, 2.5% trifluoroacetic acid, and 47.5% water.

Cocrystallization method depended on the matrix used: a) 1 μl of the bacterial cell lysate was deposited onto a sample spot of a MALDI target plate (MSP 96 target, ground steel; Bruker Daltonics, Germany) and was allowed to dry on air at room temperature. Finally, 2 μl of α-cyano-4-hydroxy cinnamic acid or ferulic acid matrix solution were deposited onto the dried sample and allowed to dry at room temperature; or b) 0.3 μl of sinapinic acid solution saturated in absolute ethanol was spotted onto a sample spot of a MALDI target plate (MSP 96 target ground steel; Bruker Daltonics, Germany) and allowed to dry at room temperature. Then, 1 μl of the bacterial cell lysate was added. Finally, 2 μl of sinapinic acid matrix were deposited onto the dried crystals and allowed to dry at room temperature. Each bacterial lysate was spotted onto the MALDI target plate in five replicas.

Table 2. Analysis of the most reproducible peaks (registration frequency equal or more than 0.7) for *E. coli* strain DH5α

Peak number	*M* (m/z)	σ (*M*)	I	σ (I)	f	M (match)	Ion type	Description
1	*4365*	*1.0*	*0.50*	*0.2*	*1*	***4364****	***M+H*$^+$**	***RL36***
						4364	M+H$^+$	UreG (fragment)
2	4777	1.61	0.13	0.05	1	4778	M+H$^+$	Q 8FKS1
						4779	M+H$^+$	Q 8FKD1
						4779	M+H$^+$	Q 1RFE4
						9553	**M+2H$^+$**	**RS20**
						9553	**M+2H$^+$**	**RL26**
3	4870	1.71	0.05	0.04	0.9	9737	M+2H$^+$	AFAF
						9737	M+2H$^+$	Q 27TL7
						9736	M+2H$^+$	Q 1R2B8
4	*5097*	*1.34*	*0.82*	*0.15*	*1*	***5096****	***M+H*$^+$**	***RS22***
						5098	M+H$^+$	LPPY
5	5150	1.56	0.08	0.03	0.9	10300	M+2H$^+$	ACYP
						10299	**M+2H$^+$**	**RS19**
						10303	M+2H$^+$	YJCB
6	5340	1.61	0.03	0.01	0.7			
7	*5381*	*1.38*	*0.64*	*0.18*	*1*	***5380****	***M+H*$^+$**	***RL34***
8	*6255*	*1.47*	*0.82*	*0.29*	*1*	***6254***	***M+H*$^+$**	***RL33** (methylated)*
9	*6316*	*1.56*	*0.38*	*0.13*	*1*	***6315****	***MH*$^+$**	***RL32***
10	*6412*	*1.57*	*0.04*	*0.02*	*0.8*	***6411****	***M+H*$^+$**	***RL30***
11	*6509*	*1.61*	*0.20*	*0.05*	*0.7*	*6507*	*M+H*$^+$	*RMF*
12	*7158*	*1.81*	*0.27*	*0.12*	*1*	***7158****	***M+H*$^+$**	***RL35***
13	*7275*	*1.69*	*0.39*	*0.17*	*1*	***7273****	***M+H*$^+$**	***RL29***
14	*7872*	*1.98*	*0.07*	*0.01*	*0.9*	***7871****	***M+H*$^+$**	***RL31***
15	*8370*	*1.93*	*0.14*	*0.06*	*1*	***8369****	***M+H*$^+$**	***RS21***
						8371	M+H$^+$	FEOA
16	8877	2.03	0.04	0.02	0.8	**8875**	**M+H$^+$**	**RL28**
17	8995	1.92	0.05	0.02	0.8	**8993**	**M+H$^+$**	**RL27**
18	*9064*	*1.98*	*0.17*	*0.07*	*1*	*9064*	*M+H*$^+$	*KOC1 (loss of Met)*
19	*9227*	*2.11*	*0.04*	*0.01*	*0.8*	*9227*	*M+H*$^+$	*KEC2*
20	*9553*	*2.09*	*0.27*	*0.06*	*1*	***9553***	***M+H*$^+$**	***RS20***
21	*9740*	*2.31*	*0.38*	*0.21*	*1*	*9737*	*M+H*$^+$	*AFAF*
						9737	*M+H*$^+$	*Q 27TL7*
						9736	*M+H*$^+$	*Q 1R2B8*
22	*10300*	*2.25*	*0.08*	*0.03*	*1*	*10300*	*M+H*$^+$	*ACYP*
						10299*	***M+H*$^+$**	***RS19***
						10303	*M+H*$^+$	*YJCB*

Peaks already reported for *E. coli* strain K-12 [20] are marked in italics. Ribosomal proteins are indicated by bold type. *M* – average value of experimental m/z for each peak (mass spectra collected from 10 independent experiments have been used); σ (*M*) – standard deviation of *M*; I–relative intensity of peak; σ (I) – standard deviation of I; f – frequency of peak registration; M (match) – value of protein mass from SwissProt/TrEMBL database (Da); *proteins' masses included in calibration list; Ion type – for matched peaks only; Description – description of matched protein according to SwissProt/TrEMBL database.

Preparation of Ribosomal Protein Fraction for MALDI Mass Spectrometry Analysis

Isolation and purification of ribosomal proteins were performed according to the protocol described earlier.[18]

Then five cycles of sonication (20 seconds at 1-minute intervals) followed by centrifugation to remove cell debris for 20 minutes were done. Briefly, ribosomes were collected by ultracentrifugation through a 10% to 30% (w/v) sucrose gradient in buffer containing 1 mmol/L HEPES (pH 7.8), 1 mmol/L $MgCl_2$, and 200 mmol/L NH_4Cl, over 18 hours at 23,000 rpm. The 30S and 50S peak fractions were collected separately pelleted by ultracentrifugation (20 hours at 45,000 rpm) and resuspended in buffer 1 mmol/L HEPES (pH 7.8), 1 mmol/L $MgCl_2$, and 200 mmol/L NH_4Cl. All procedures were conducted at 4°C. During the preparation extreme care was taken to prevent ribonuclease contamination. Therefore, only glassware pre-baked at 180°C for at least 2 hours, or sterile plastic tubes and pipette tips were used.

Purified protein solution (2 μl) was spotted onto a MALDI target plate (MSP 96 target, ground steel; Bruker Daltonics, Germany) and was allowed to air dry at room temperature. Finally, 2 μl of α-cyano-4-hydroxy cinnamic acid matrix solution were deposited onto the dried sample and allowed to dry at room temperature.

Mass Spectrometry Analysis

Mass spectra profiles were acquired using a Microflex LT MALDI-ToF mass spectrometer (Bruker Daltonics, Germany) equipped with a N_2 337 nm laser. The molecular ions were measured in the linear positive ion mode with instrument parameters optimized for the range of 2000 to 20,000 m/z. The laser intensity was set slightly above the desorption and ionization threshold of the sample.

E. coli DH5α was used for calibration and instrument parameter optimization. Precisely, there were masses matched to RL36, RS22, RS34, RL32, RL30, RL35, RL29,

RL31, RS21, and RS19 (see Table 2) included in the calibration list. A three-step calibration procedure was performed. First, the *x*-axis was calibrated using mentioned above masses of reproducible and high resolution proteins included in mass control list (internal calibration). The calibration constant of *E. coli* DH5α was applied to the measurement of experimental strains. Further spectra examination was performed using the FlexAnalysis 2.4 software (Bruker Daltonics, Germany).

Four different mass spectra from each replicate were acquired resulting in 20 spectra per sample (four spectra for each of five replicas). Five hundred individual laser shots were accumulated for each mass spectrum. Spectra quality criteria were: high spectra reproducibility, little noise and a flat baseline (signal-to-noise ratio above 15), presenting as much peaks as possible and an average resolution of 600 within the range of m/z selected for analysis. Both manual and automatic measurements were applied. The mass accuracy after external calibration using known *E. coli* protein signals was about 200 ppm.

Spectra processing for visual inspection was performed using the FlexAnalysis 2.4 software: peak lists were calculated using the centroid peak algorithm, with signal-to-noise ratio set at 15 and relative intensity threshold set at 10%.

For matching the peaks of interest, their m/z values were compared against the SwissProt/TrEMBL database (Expasy, Swiss Bioinformatics Institute) by manual search using the Sequence Retrieval System module at *http://www.expasy.ch/srs5/* of the definite bacteria. The only parameters used in the search were ≪Organism≫ and ≪MolWeight≫ (experimental mass allowing for a mass window) corresponding to an experimental mass accuracy of 300 ppm. When no database protein was found in the mass window, the search was repeated with a mass value corresponding to N-terminal methionine loss, which is the most common protein modification in bacteria.

To construct and compare the characteristic reference spectra (main spectra) of certain strains, the mass spectra acquired (20 raw spectra per sample) were processed with the MALDI Biotyper 1.1 software (Bruker Daltonics, Germany). The main spectra were constructed based on processed raw spectra after smoothing, baseline subtraction, normalization, and peak picking. The resulting twenty individual peak lists per strain were used to construct a main spectrum, which contained the average peak position and peak intensities and their frequency in the set of spectra for the most prevalent peaks. All processing steps were performed automatically using the default settings of the software. Thereby, a main spectrum displayed the most reproducible peaks typical for a certain bacterial strain. Cluster analysis was performed based on comparison of strain specific main spectra created as described above. In a first step, each main spectrum of the dataset was compared with each other main spectrum resulting in a matrix of cross-wise identification scores. This matrix was used to calculate distance values for each pair of main spectra. Based on these values a dendrogram was generated using the according function of the statistical toolbox of Matlab 7.1 (The MathWorks Inc.) integrated in the MALDI Biotyper software. The parameter settings were: "Distance Measure = Euclidian" and "Linkage = complete." The linkage function is normalized according to the distance between 0 (perfect match) and 1000 (no match).

Extraction of Genomic DNA, Amplification, and Sequencing of Neisseria *Genes*

Bacterial DNA was isolated according to Boom et al.[19] *N. gonorrhoeae* genes *rpmE, rpmH, rpmJ* and *N. meningitidis* genes *rpsT, rpmA* were amplified with followed primer sets: *rpmE*-f 5′- TTTTATCGAATATCCTCTTGACG-3′, and *rpmE*-r 5′-GGCTTCGTCGCCTTGTCC-3′; *rpmH*-f 5′-CCGATTATACCGACTCGCCG-3′, and *rpmH*-r 5′-CGCGTTTCATATAGTTCCGCTC-3′; *rpmJ*-f 5′-ATATTTCTGGGAAGATGCGG-3′, and *rpmJ*-r 5′-AATTTAGCACGAGTAGCACC-3′; *rpsT*-f 5′-GCACCCTTTCCGCCATTTC-3′, and *rpsT*-r 5′-CCGTTTTTTTGCGCTGTCAG-3′; *rpmA*-f 5′-GGCTAAAGTAGTGGCACACG-3′, and *rpmA*-r 5′-TGAAAAGATGCGGCTTCAGG-3′. All primers were designed using the Oligo_6.37 software (Molecular Biology Insights Inc.) and sequences of *N. gonorrhoeae* strain FA 1090 (NC_002946), *N. meningitidis* strains FAM18, serogroup C (NC_008767), MC58, serogroup B (NC_008767), Z2491, serogroup A (NC_003116).

PCR amplification was performed in a reaction mixture containing 66 mM Tris-HCl (pH 9.0), 16.6 mmol/L $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 250 μM of each dNTP, 1U of TaqDNA polymerase (Promega), and 10 pmol of each primer in final volume 10 μl using the thermocycler DNA Engine Tetrad 2 (MJ Research). The products of amplification were analyzed in 2% agarose gel.

Then, 0.5 U of shrimp alkaline phosphatase (Promega), and 5 U Exonuclease I (Fermentas) were added to each PCR product and incubated at 37 °C for 30 minutes for dephosphorylation of the 5′-end phosphate groups of deoxynucleoside triphosphates and primer elimination. Subsequently, the enzymes were inactivated by heating at 85 °C for 10 minutes.

Sequencing was performed using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit and the ABI Prism 3100 Genetic Analyser (Applied Biosystems; Hitachi, Japan).

The amino acid sequences of *N. gonorrhoeae* ribosomal proteins RL31, RL34, RL36, and *N. meningitidis* ribosomal proteins RS20, RL27 were deduced from the nucleotide sequences of corresponding genes *rpmE, rpmH, rpmJ,* and *rpsT, rpmA,* respectively, using the Vector NTI Suite v.9.0 software (InforMax Inc., USA).

Results

In our investigation, the initial DBP experiments were started with already well-characterized bacteria such as *E. coli*. In the well-studied case of *E. coli* MALDI-TOF profiling, about 50% of the observed peaks could be

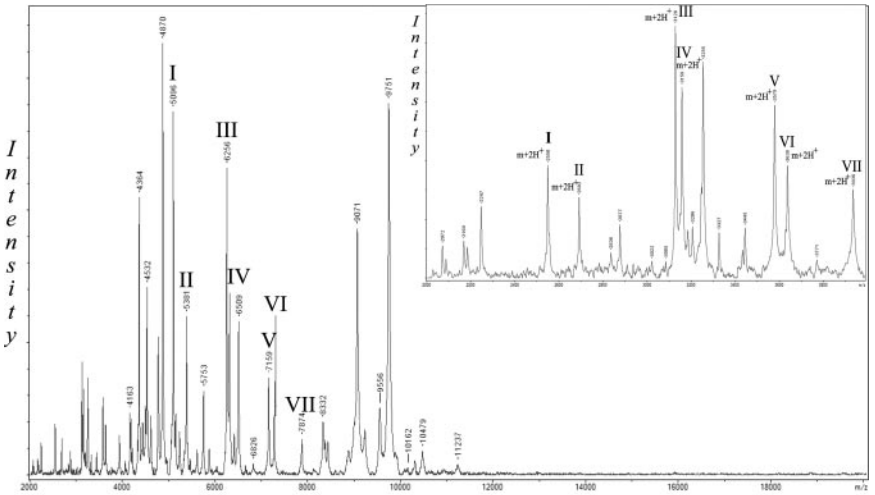

Figure 1. MALDI-TOF mass spectrum of a whole-cell extract of *E. coli* strain DH5α. The mass-spectrum was recorded within the range of m/z 2000 to 20,000, zoomed region (m/z 2000 to 4000) illustrates peaks of doubly charged ions. The roman numerals indicate signal pairs of singly and doubly charged ions of the same molecules. The relative intensities of the ions are shown on the *y* axis, the mass to charge ratios are shown on the *x* axis.

assigned to ribosomal proteins, which are known to account for more than 20% of total cell protein content.[20] Because ribosomal proteins are relatively basic, they can be well extracted by acidic solutions. In addition, these positively charged proteins can be measured with high sensitivity in the positive MALDI-TOF measurement mode.

Different acetonitrile and trifluoroacetic acid concentrations for preparation of whole-cell lysates and protein extraction were tested. The best results were obtained using 50% acetonitrile/2.5% trifluoroacetic acid /47.5% water solution. Three different MALDI matrices have been examined (for more details see "Material and Methods"). For further work, α-cyano-4-hydroxy cinnamic acid matrix was selected because of the best mass spectra quality (ie, resolution, signal-to-noise ratio, number of peaks, intensity, and mass range of detected proteins) and the

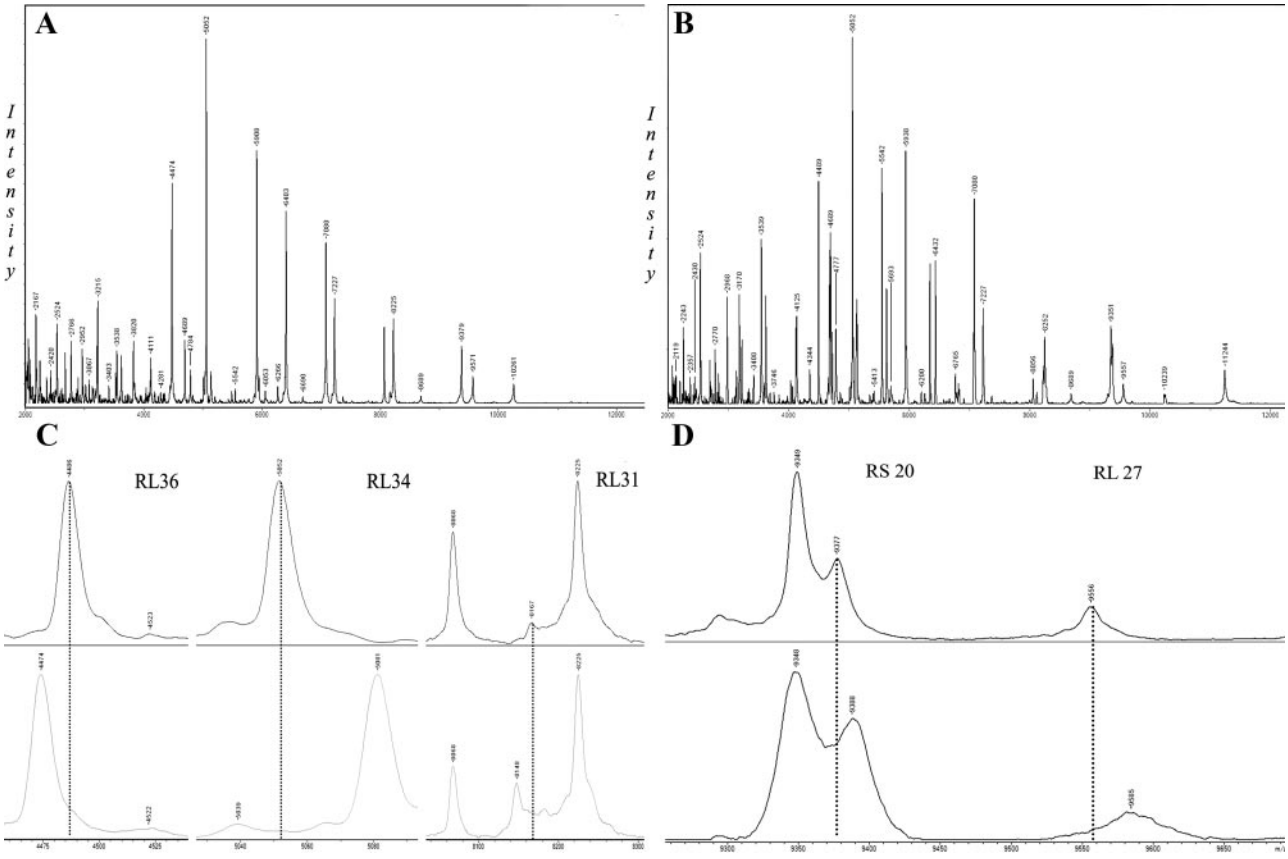

Figure 2. MALDI-TOF mass spectra of whole-cell extracts of pathogenic *Neisseria* strains*: N. gonorrhoeae* strain ATCC 49226 (**A**) and *N. meningitidis* clinical strain 537 (**B**). The relative intensities of the ions are shown on the Y axis, the mass to charge ratios on the *x* axis. Changes of MALDI-TOF mass spectra observed for clinical strains of *N. gonorrhoeae* (**C**) and *N. meningitidis* (**D**). Variants of peaks corresponding to ribosomal proteins RL 36, RL 34, and RL 31 of *N. gonorrhoeae* and of those corresponding to ribosomal proteins RS 20 and RL27 of *N. meningitidis* are shown.

Table 3. Comparative Analysis of the Most Reproducible Peaks (Registration Frequency Equal or More than 0.7) for *N. gonorrhoeae* and *N. meningitidis* Strains

Neisseria gonorrhoeae ATCC 49226									*Neisseria* meningitidis 537 (serogroup B)								
Peak number	*M*	s (*M*)	I	σ (I)	f	*M* (match)	Ion type	D	Peak number	*M*	σ (*M*)	I	σ (I)	f	*M* (match)	Ion type	D
1	**4474**	**1.04**	**0.66**	**0.07**	**1**	**4473**	**M+H$^+$**	**RL36**									
									1	**4489**	**0.52**	**0.70**	**0.25**	**0.9**	**4489**	**M+H$^+$**	**RL36**
2	4511	1.71	0.04	0.01	0.8												
3	***4689***	***1.17***	***0.37***	***0.24***	***1***	***9377****	***M+2H$^+$***	***RS20***	***2***	***4688***	***0.26***	***0.35***	***0.21***	***0.7***	***9377****	***M+2H$^+$***	***RS20***
4	4784	1.22	0.15	0.08	1	9570	M+2H$^+$	RL27									
5	5010	1.09	0.08	0.03	1	5010											
6	***5052***	***1.2***	***1***	***0***	***1***	***5051***	***M+H$^+$***	***RL34***	***3***	***5051***	***0.03***	***0.77***	***0.24***	***1***	***5051***	***M+H$^+$***	***RL34***
									4	5115	0.36	0.25	0.11	0.7	10230*	M+2H$^+$	RS19
7	***5130***	***1.28***	***0.21***	***0.13***	***0.9***	***10259****	***M+2H$^+$***	***RS19***	*5*	*5129*	*0.53*	*0.31*	*0.17*	*0.8*			
8	5484	1.33	0.03	0.01	0.7												
									6	5541	0.35	0.57	0.05	0.9			
									7	5624	0.18	0.38	0.14	0.7			
9	**5908**	**1.4**	**0.72**	**0.06**	**1**	**5907**	**M+H$^+$**	**RL33**									
									8	**5937**	**0.46**	**0.47**	**0.18**	**1**	**5937**	**M+H$^+$**	**RL33**
10	5946	1.31	0.10	0.03	1												
11	6053	1.54	0.03	0.01	0.9												
									9	**6342**	**0.70**	**0.32**	**0.15**	**1**	**6342**	**M+H$^+$**	**RL32**
12	**6404**	**1.49**	**0.55**	**0.04**	**1**	**6402***	**M+H$^+$**	**RL32**									
									10	6431	0.50	0.47	0.29	0.9			
13	***7080***	***1.59***	***0.66***	***0.15***	***1***	***7078***	***M+H$^+$***	***RL29***	***11***	***7078***	***0.52***	***0.55***	***0.24***	***1***	***7078***	***M+H$^+$***	***RL29***
14	***7227***	***1.65***	***0.32***	***0.05***	***1***	***7226****	***M+H$^+$***	***RL35***	***12***	***7226***	***0.51***	***0.25***	***0.07***	***0.7***	***7226****	***M+H$^+$***	***RL35***
15	8068	1.81	0.28	0.06	1												
16	**8167**	**1.95**	**0.06**	**0.03**	**1**	**8165**	**M+H$^+$**	**RL31**									
17	**8225**	**1.81**	**0.49**	**0.25**	**1**	**8224***	**M+H$^+$**	**RS21**									
									13	9350	1.15	0.24	0.08	0.7			
18	***9379***	***1.97***	***0.28***	***0.13***	***1***	***9377****	***M+H$^+$***	***RS20***	***14***	***9377***	***2.35***	***0.19***	***0.09***	***0.9***	***9377****	***M+H$^+$***	***RS20***
									15	9557	0.53	0.31	0.17	0.8	9554*	M+H$^+$	RL27
19	**9570**	**2.17**	**0.1**	**0.04**	**1**	**9568***	**M+H$^+$**	**RL27**									
									16	**10237**	**1.78**	**0.06**	**0.04**	**0.7**	**10237***	**M+H$^+$**	**RS15**
20	**10260**	**2.13**	**0.09**	**0.05**	**1**	**10259***	**M+H$^+$**	**RS19**									
									17	11248	0.50	0.47	0.29	0.9			

Identical peaks are marked by italics. Ribosomal proteins are indicated by bold type. M – average value of experimental m/z for each peak (mass spectra collected from 10 independent experiment have been used); σ (M) – standard deviation of M; I – relative intensity of peak; σ (I) – standard deviation of I; f – frequency of peak registration; M (match) – value of protein masses from SwissProt/TrEMBL database (Da); * with N-terminal methionine lost; Ion type – for matched peaks only; D – description of matched protein according to SwissProt/TrEMBL database.

matrix crystals homogeneity allowing fast automated measurements of high quality (data not shown).

A MALDI-TOF mass spectrum of *E. coli* strain DH5α whole-cell lysate is shown in Figure 1. Visual review showed that mainly the mass range up to 12000 m/z is informative and the majority of peaks within 2000 to 4000 m/z range correspond to doubly charged ions. In addition, some doubly charged ions were located within the 4000 to 5000 m/z region. However, the majority of these peaks were reproducible and of good resolution (higher than 500). Thus, further bioinformatic mass spectra analyses were performed within the 2000 to 12000 m/z range.

Peak lists of 10 mass spectra collected from three different cultures of *E. coli* strain DH5α were generated. The most reproducible peaks (registration frequency is equal or more than 0.7) with SwissProt/TrEMBL database searching results are listed in Table 2. Maximum mean-square deviation found was 2.6 Da for m/z value 10,300 Da and 0.29 for relative intensity. The peak list obtained for *E. coli* DH5α was similar to the one for *E. coli* K-12 described earlier.[21] Highly reproducible peaks (registration frequency is 1.0) were used as calibrators in further DBP tests.

The technique optimized for DBP analysis of *E. coli* was applied to study bacteria of the genus *Neisseria*, starting with *N. gonorrhoeae* laboratory strain ATCC 49226 and *N. meningitidis* clinical strain 537, whose mass spectra are shown on Figure 2A, B).

Spectra quality and reproducibility for *N. gonorrhoeae* cells were similar to *E. coli*. The described protocol allowed us to obtain good quality MALDI mass spectra for *N. gonorrhoeae* laboratory strain ATCC 49226, which are much richer in peaks, and thereby more informative, than analogous data reported previously.[22] Maximum mean-square deviation was shown as 2.8 Da for m/z value 9570 and 0.24 for relative intensity. Results of accuracy and reproducibility analysis for 10 different spectra (corresponding to two different cultivations of *N. gonorrhoeae* strain ATCC 49226) are summarized in Table 3. Generally, the mass spectra contained more than 70 peaks, with 20 of them being the most reproducible (registration frequency equal or more than 0.7). As expected most of these peaks could be matched to ribosomal proteins, a finding that was confirmed by analysis of protein signals from a purified ribosomal fraction of the cell lysate (Figure 3).

In contrast to *N. gonorrhoeae*, mass spectra of the closely related species *N. meningitidis* (clinical strain 547) exhibited only 47 significant peaks; 17 were found to be the most reproducible ones. Despite taxonomical (phylogenetic) relationship between *N. gonorrhoeae* and *N. meningitidis*, only six peaks were found identical for these species (selected by gray in Table 3).

To reveal the heterogeneity of strains under investigation Por-typing of *N. gonorrhoeae* and serotyping of *N. meningitidis* was performed (Table 1). Commonly, *N.*

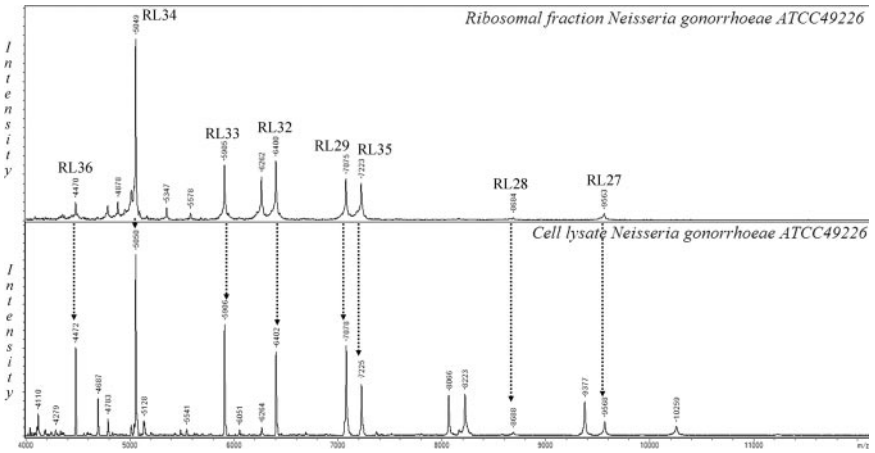

Figure 3. MALDI-TOF mass spectra of the purified ribosomal fraction and cell lysate of *N. gonorrhoeae* ATCC 49226 strain. The relative intensities of the ions are shown on the *y* axis, and the mass to charge ratios are shown on the *x* axis.

gonorrhoeae strains were distributed among 10 different *por*-types. Five of the investigated isolates were found to belong to the PorB1a serovar (PIA); the remaining 24 strains were assigned to serovar PorB1b. For *N. meningitidis* strains, all three serogroups were revealed, but serogroup A was predominant.

All together, MALDI-TOF mass spectra were collected for 29 clinical strains of *N. gonorrhoeae*, 13 clinical strains of *N. meningitidis* and 15 strains of non-pathogenic *Neisseria* (listed in Table 1). Comparative analysis of peak lists showed good reproducibility within each species with slight differences between strains.

Thus, for *N. gonorrhoeae* the majority of significant peaks were constant and only three peaks m/z 4473, 5051, and 8165 changed their m/z value to 4487, 5081, and 8146 respectively for several strains (Figure 2C). For *N. meningitidis* strains similar changes of two peaks with m/z 9377, and 9557 (transformed to 9393 and 9587, respectively) have been observed (Figure 2D).

For *N. gonorrhoeae* strains, it was possible to unveil the molecular basis of the observed changes. The variable peaks were matched to ribosomal proteins RL36, RL34, and RL31, respectively. Full lengths of the corresponding *rpmJ, rpmH,* and *rpmE* genes were sequenced for strains with the original and modified mass profile. A single nucleotide alteration leading to an amino acid substitution was found for each gene. The expected mass differences calculated from the amino acid substitutions were coincident with the observed mass changes (Table 4). These findings confirmed the results of SwissProt/TrEMBL database search and supported previous reports that mainly ribosomal proteins contribute to the MALDI-TOF profiles of bacteria in the mass range that we have investigated. Based on m/z values of these peaks the *N. gonorrhoeae* strains were divided into four different groups with m/z peaks' combinations 4473/5051/8165; 4487/5051/8165; 4487/5051/8146; and 4473/5081/8165. It should be mentioned that most clinical strains as well as strain ATCC 49226 belonged to the first group corresponding to the wild-type sequence.

For *N. meningitidis* strains, the variable peaks were matched to ribosomal proteins RS20 and RL27. Full-length sequencing of the corresponding genes *rpsT,* and *rpmA* was performed. As for *N. gonorrhoeae* strains, the nucleotide alterations leading to amino acid substitutions were found, and the theoretically calculated mass differences for these proteins were coincident with the mass changes observed (Table 4). In the case of *N. meningitidis,* only two m/z combinations of variable peaks were found—9377/9557 and 9393/9587.

While the profiles obtained for *N. gonorrhoeae* and *N. meningitidis* showed only limited intraspecies variability

Table 4. Molecular Changes of Ribosomal Proteins Corresponding to Observed Differences within *N. gonorrhoeae* and *N. meningitidis* MALDI-TOF Mass Spectra Groups

m/z	Matched protein (SwissProt/TrEMBL)	Gene	Nucleotide changes	Amino acid changes	Calculated mass changes, Da
N. gonorrhoeae					
4473	RL36	*rpmJ*	*wt	wt	4473
			G19→A	V7→I	4487
5051	RL34	*rpmH*	Wt	wt	5051
			G109→A	A37→T	5081
8166	RL31	*rpmE*	Wt	wt	8165
			G29→A	R10→H	8146
N. meningitides					
9378	RS20	*rpsT*	Wt	wt	9378
			G169→A	V57→I	9393
9557	RL27	*rpmA*	Wt	wt	9554
			G79→A	G27→S	9584

* wt – nucleotide and amino acid sequences are corresponding to those from *N. gonorrhoeae* strain FA 1090 (NC 002946) and *N. meningitidis* strains FAM18, serogroup C (NC 008767), MC58, serogroup B (NC 008767), Z2491, serogroup A (NC 003116), respectively.

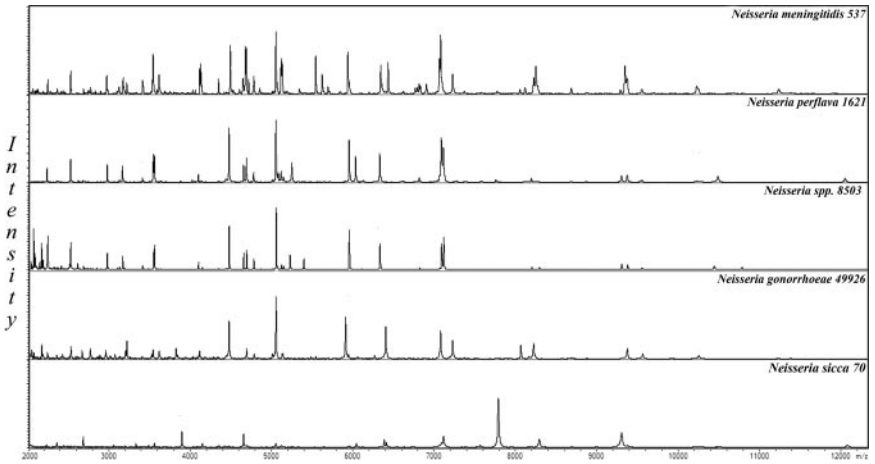

Figure 4. MALDI-TOF mass spectra comparison of closely related species of *N. gonorrhoeae*, *N. meningitidis*, *N. sicca*, *N. preflava*, and non-determined *Neisseria* spp 8503. The relative intensities of the ions are shown on the Y axis, and the mass to charge ratios are shown on the *x* axis. The patterns display the individual distinguishes among isolates that lead to *Neisseria* species separation.

comparison of MALDI-TOF mass spectra collected for *N. gonorrhoeae*, *N. meningitidis* and non-pathogenic *Neisseria* species had numerous significant differences in peak positions (Figure 4). For further cluster analysis, a database containing the main spectra of the 57 *Neisseria* strains was created using the MALDI Biotyper 1.1 software. A main spectrum based on the comparative analysis of twenty raw spectra was generated for each strain using the dedicated data processing algorithms of the software.

The established database was used to examine specificity of the technique for the identification of the different *Neisseria* species. Alignment of all 57 main spectra divided them into three separate groups corresponding to *N. gonorrhoeae*, *N. meningitidis* and non-pathogenic *Neisseria* (Figure 5) that demonstrated the possibility of isolates identification at the species level.

To investigate if different MALDI-TOF mass spectrometers can be used for bacteria species identification, two different sets of mass spectra collected by means of a benchtop microflex MALDI-TOF mass spectrometer and an ultraflex II MALDI-TOF/TOF mass spectrometer were compared (Table 5). We found a good correlation (more than 0.86) between the mass spectra collected by the different devices for *E. coli*, *N. gonorrhoeae*, *N. meningitides,* and non-pathogenic *Neisseria* strains.

In addition, a separate MALDI Biotyper database containing 1671 main spectra generated from different environmental and clinically relevant microorganisms was used to show the specificity of pathogenic *Neisseria* species identification. *N. gonorrhoeae* and *N. meningitidis* isolates could be identified unambiguously on the species level regardless of the instrument used for the profile spectra acquisition.

Discussion

Advantages of MALDI-TOF mass spectrometry make this technique very attractive for the rapid identification of bacteria based on the simultaneous detection of a mixture of cellular components, mainly proteins and pep-

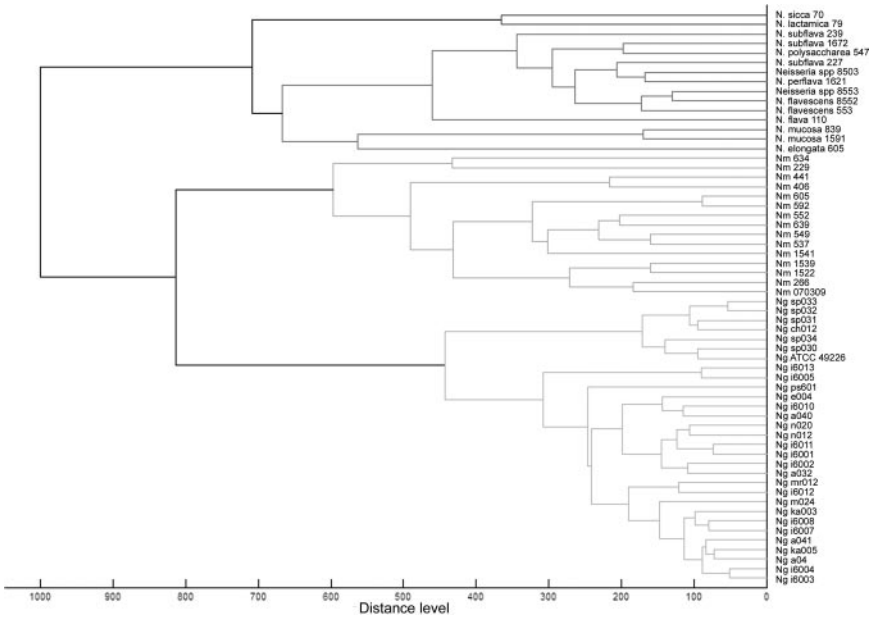

Figure 5. A score oriented dendrogram of MALDI-TOF mass spectra profiles was generated using the MALDI Biotyper 1.1 software with the following settings for peak picking: lower bound 3000, upper bound 11,000, maximum peaks 100 and threshold 0.001. *Neisseria* species are listed in Table 1. The dendrogram was generated with the following settings: the distance measure was set at Euclidian and linkage set at completion.

Table 5. Comparison of MALDI Mass Spectra

Np (U) 14	0.00	0.00	0.09	0.09	0.05	0.05	0.18	0.18	0.15	0.15	**0.93**	**0.98**
Np (M) 13	0.00	0.01	0.23	0.23	0.16	0.16	0.36	0.36	0.30	0.30	**0.97**	
Nm2 (U) 10	0.00	0.02	0.73	0.81	0.70	0.74	0.87	0.87	**0.93**	**0.93**		
Nm2 (M) 9	0.00	0.00	0.73	0.81	0.70	0.74	0.87	0.87	**0.93**			
Nm1 (U) 8	0.00	0.00	0.64	0.73	0.60	0.64	**0.97**	**0.97**				
Nm1 (M) 7	0.00	0.00	0.67	0.73	0.60	0.64	**0.97**					
Ng2 (U) 6	0.00	0.00	0.80	0.92	**0.89**	**0.99**						
Ng2 (M) 5	0.00	0.00	0.88	0.83	**0.98**							
Ng1 (U) 4	0.00	0.00	**0.86**	**0.99**								
Ng1 (M) 3	0.00	0.00	**0.95**									
Ec (U) 2	**0.88**	**0.91**										
Ec (M) 1	**0.99**											
	EC (M) 1	EC (U) 2	Ng1 (M) 3	Ng1 (U) 4	Ng2 (M) 5	Ng2 (U) 6	Nm1 (M) 7	Nm1 (U) 8	Nm2 (M) 9	Nm2 (U) 10	Np (M) 13	Np (U) 14

E. coli (EC) strain DH5α, *N. gonorrhoeae* strains ATCC 49226 (Ng1) and ps601 (Ng2), *N. meningitidis* strains *537* (Nm1) and 229 (Nm2), and non-pathogenic *N. subflava 227* (Np) strain. We used a microflex LT MALDI-TOF mass spectrometer (M) and an ultraflex II MALDI-TOF/TOF mass spectrometer (U) from Bruker Daltonics, Germany. The composite correlation index (CCI) matrix was calculated using the MALDI Biotyper 1.1 software with the following settings: lower bound 3000, upper bound 12000, resolution of the mass range 4, number of intervals for CCI 4. CCIs for comparison mass spectra are indicated by bold.

tides, directly from "whole" cells without preliminary extraction and separation. The current trend of this approach to introduce it into routine is finding the balance between simplicity, speed of analytical procedure and optimal characteristics of the obtained mass spectra required for particular applications.

The study presented is focused on features of DBP by MALDI TOF mass spectrometry for investigation of clinically relevant microorganisms such as *Neisseria*. Interest in *N. meningitidis* and *N. gonorrhoeae* remains permanent because of their well-known contribution in the structure of human infection diseases. The annual local outbreaks of meningitis and the spreading of multidrug resistant gonococci require the development the new effective molecular systems for species identification and typing. Mass spectrometry based methods seem to be most promising in this field. Recently, Oliver Schmid et al, 2005, used surface enhanced laser desorption/ionization-time of flight mass spectrometry in combination with artificial neural networks for the identification of *N. gonorrhoeae*.[23] In addition the automated comparative sequence analysis by base-specific cleavage and mass spectrometry was used for *N. meningitidis* typing.[24]

The challenge of our investigation was to apply a MALDI-TOF mass spectrometry technique for species identification of closely related bacteria from the genus *Neisseria*. This approach based on mass spectral profile analysis, which does not require any sophisticated purification of bacterial proteins, enables species identification with significantly lower costs than surface enhanced laser desorption/ionization-time of flight mass spectrometry without any loss of efficiency. Some years ago, a similar approach was shown to be suitable for investigation of different *Haemophilus* spp including *N. gonorrhoeae* as a microorganism that exhibits a similar biochemical and bacteriological profile.[25] In our case, the DBP technique was extended by using correlation analysis to compare mass spectra acquired with different instruments. The high mass spectra reproducibility together with low intraspecies variability and evident interspecies heterogeneity indicated the applicability of this approach for *Neisseria* species identification. Moreover, analysis of *N. gonorrhoeae* ribosomal fraction confirmed that most of the peaks registered using the described protocol belong to ribosomal proteins, conserved for certain bacterial species.

Furthermore, we evaluated the DBP approach as a rapid tool useful for identification of bacterial isolates. The following steps of the DBP protocol investigation and optimization were performed: theoretical analysis of earlier accumulated experience, adjustment of the sample preparation protocol using *E. coli* to compare our results with data of other studies (matrix, extraction solution composition, etc), adjustment of the sample preparation protocol and MALDI-TOF mass spectrometry procedure using first references and then clinical strains of the investigated bacterial species. An important point is that the clinical strains were epidemiologically different strains covering interspecies variability in the investigated *Neisseria* species.

The problem of the *Neisseria* genus study is complex. Although the clinically important microbes—*N. gonorrhoeae* and *N. meningitidis*—are relatively straightforward to identify, the differences between many of the non-pathogenic strains are small and the speciation of these strains within a diagnostic setting is not always possible. While the identification of non-pathogenic *Neisseria* to species level is generally not required, the misidentification of these strains as *N. gonorrhoeae* or *N. meningitidis* can have serious health, legal and social consequences.

Based on the described experiments we can conclude that the suggested DBP technique works very well for species identification of *N. gonorrhoeae* and *N. meningitidis.* Demonstrated performance of the MALDI profiling approach is similar to recently published data describing the capability of analogue techniques for species identification of closely related mycobacteria[26,27] and for Gram-negative non-fermenting bacteria.[8]

As far as non-pathogenic *Neisseria* are concerned, their mass spectral profiles are completely different from *N.*

gonorrhoeae and *N. meningitidis* (distance level is 1000) and form a rather heterogenic group. Probably, further enlargement of this group by introduction of more commensally *Neisseria* allows obvious species discrimination as well.

It should be mentioned that despite investigated *N. gonorrhoeae* and *N. meningitidis* strains belonged to different genotypes or serotypes, little intraspecies heterogeneity has been found looking at the prominent peaks of the spectra. The four main proteotypes described for*N. gonorrhoeae* and two for *N. meningitidis* did not reflect the true variability of these microorganisms. Therefore, for *Neisseria* genus the described DBP approach seems more suitable for robust species identification in contrast to resequencing techniques, eg, MALDI-TOF mass spectrometry based multilocus sequence typing,[24] used for strain differentiation. But the capability of subtyping using low-intensity peaks, eg, in a hierarchical approach after speciation by standard pattern matching, has to be evaluated.

On the other hand, species identification by means of DBP is much easier, cheaper and more rapid than by using DNA-analysis based technologies. The optimized measuring protocol based on the strong criteria of mass spectra collection (high spectra reproducibility, little noise and a flat baseline, presenting as many peaks as possible and medium resolution of 600 within the range of m/z selected for analysis) enabled fast acquisition of high-quality spectra in automatic measurements. In general, the MALDI mass spectra produced by our methodology were conserved for each specimen and slightly different within the same species. Moreover, most differences observed comparing individual measurements of one strain were in intensities, and only in few cases in absence or presence of peaks. These measurement variations, which are intrinsic to the MALDI-TOF technology, are reduced significantly by merging 20 raw spectra for each sample into one main spectrum, which displays the most reproducible peaks. Nevertheless, it is important to keep experimental conditions constant to guarantee a secure species identification.

At the same time, the approach has great advantages such as the requirement of a small amount of biological material, very easy sample preparation, highly accurate measurement, and low cost per analysis. As a whole-cell fingerprinting technique and due to the low amount of material required, DBP allows the rapid identification of large numbers of microbial isolates at a very early stage in the screening process, eg, from bacterial colonies growing on pre-isolation plates.

Thus, we conclude that the DBP approach using the described rapid, simple and effective protocol coupled with special software, eg, the MALDI Biotyper, equipped with optimized algorithms for identification and classification is highly suitable for the secure *Neisseria* species identification in routine settings. Further studies will be dedicated to investigations of other bacterial genera and to the utilization of the minor spectra variability between different subtypes for subspecies classification.

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