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Rapid Screening of Epidemiologically Important *Salmonella enterica* subsp. *enterica* Serovars by Whole-Cell Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry[▽]

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Currently, 2,610 different *Salmonella* serovars have been described according to the White-Kauffmann-Le Minor scheme. They are routinely differentiated by serotyping, which is based on the antigenic variability at lipopolysaccharide moieties (O antigens), flagellar proteins (H1 and H2 antigens), and capsular polysaccharides (Vi antigens). The aim of this study was to evaluate the potential of matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry for rapid screening and identification of epidemiologically important *Salmonella enterica* subsp. *enterica* serovars based on specific sets of serovar-identifying biomarker ions. By analyzing 913 *Salmonella enterica* subsp. *enterica* strains representing 89 different serovars using MALDI-TOF mass spectrometry, several potentially serovar-identifying biomarker ions were selected. Based on a combination of genus-, species-, subspecies-, and serovar-identifying biomarker ions, a decision tree classification algorithm was derived for the rapid identification of the five most frequently isolated *Salmonella enterica* serovars, Enteritidis, Typhimurium/4,[5],12:i:-, Virchow, Infantis, and Hadar. Additionally, sets of potentially serovar-identifying biomarker ions were detected for other epidemiologically interesting serovars, such as Choleraesuis, Heidelberg, and Gallinarum. Furthermore, by using a bioinformatic approach, sequence variations corresponding to single or multiple amino acid exchanges in several biomarker proteins were tentatively assigned. The inclusivity and exclusivity of the specific sets of serovar-identifying biomarker ions for the top 5 serovars were almost 100%. This study shows that whole-cell MALDI-TOF mass spectrometry can be a rapid method for prescreening *S. enterica* subsp. *enterica* isolates to identify epidemiologically important serovars and to reduce sample numbers that have to be subsequently analyzed using conventional serotyping by slide agglutination techniques.

Salmonella is a major zoonotic food-borne pathogen, causing outbreaks and sporadic cases of gastroenteritis in humans worldwide (24). Two species are currently recognized in the genus *Salmonella*, *Salmonella enterica* and *Salmonella bongori*. *S. enterica* has been further subdivided into six subspecies (48), of which *Salmonella enterica* subsp. *enterica* is the most frequently isolated and is associated with gastrointestinal disease in a wide range of mammalian hosts. Traditionally, a number of biochemical reactions differentiate *Salmonella* species and subspecies (16). The *Salmonella* serotyping scheme according to White-Kauffmann-Le Minor is accepted worldwide as a gold standard for the differentiation of salmonellae below the subspecies level (19). It is based on a combination of biochemical reactions and serotyping of the somatic O, flagellar H, and capsular Vi antigens. However, serotyping, which is usually performed by slide agglutination, is a laborious, time-intensive, and expensive method requiring more than 250 different antisera.

Whole-cell matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) based on intact protein profiling, a technique also known as direct bacterial profiling, has increasingly been studied for bacterial species identification in recent years (1, 8, 12, 13, 13, 29, 34, 35, 45). Reproducibility issues had been a major concern, but it was shown that the technique provides reliable results despite some experimental and biological variabilities that are inherent to such a phenotypic approach (14, 41, 49, 51, 54–56). A sufficient number of stable mass signals of major housekeeping proteins, mainly ribosomal proteins, can reproducibly be detected and used for bacterial species identification by using simple mass pattern-matching approaches or more sophisticated algorithms to compare and estimate the similarities between spectra (3, 9, 22, 23, 26, 27, 29, 37, 43). These approaches don't rely on actual identification of the biomarker ion peaks in an MS spectrum but on the characteristic mass profile generated by a set of ion peaks that constitute a bacterial "fingerprint." While the majority of these studies described the usage of the technique for species identification, bacterial characterization below the species level, e.g., for discrimination of serovars, was rarely approached (4, 28, 33, 43, 46). Recently, we optimized and evaluated the use of whole-cell MALDI-TOF mass spectrometry for *Salmonella* identification and differentiation on the subspecies level (13). Here, we extended the analysis for *Salmonella enterica* subsp. *enterica* (subspecies I) in order to study the suitability of the technique for differentiation of salmonellae at the serovar level. Generally, only a few serovars represent the majority of isolates from humans and animals. For example, in Europe the 10 most frequently isolated *Salmonella* serovars in humans include serovars Enteritidis, Typhimurium, Virchow, Infantis, and Hadar and accounted for

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more than 85% of the confirmed salmonellosis cases in humans during the years 2006 and 2007. Serovar Enteritidis was by far the predominant serovar (67.8%), followed by serovar Typhimurium (15.6%) (2).

The aim of this study was to establish and validate a rapid MALDI-TOF MS-based method that allows *Salmonella* identification at the species, subspecies, and serovar levels at once. As a proof of principle, the study focused on the recognition and validation of serovar-identifying biomarker ions for the frequently isolated *Salmonella* subsp. *enterica* serovars Typhimurium, Enteritidis, Virchow, Infantis, and Hadar, enabling rapid screening and identification.

MATERIALS AND METHODS

Bacterial strains. Altogether, 913 *Salmonella enterica* subsp. *enterica* strains were selected for this study. A strain list is shown in Table 1. All strains were biochemically differentiated on the subspecies level (30–32) and serotyped by slide agglutination with O antigen-specific and H antigen-specific sera (Sifin Diagnostics, Germany, Berlin) according to the White-Kauffmann-Le Minor scheme (19). The strain collection represents epidemiologically unlinked strains originating from different regions and herds in Germany that were received at the National Reference Laboratory for Salmonella, Berlin, Germany, on a routine basis for serotyping. The strains selected were frequently isolated serovars in humans and animals and included host-restricted and host-adapted serovars, as well as more rare serovars displaying similar antigenic formulas.

Cell culturing and preparation of samples for whole-cell MALDI-TOF MS. The strains were grown at 37°C for 24 h ± 1 h on Mueller-Hinton agar (Oxoid, Greve, Denmark). Bacteria were removed from agar plates by using a sterile pipette tip and applied directly as a thin film onto a 384-position MALDI sample target (Bruker Daltonics, Bremen, Germany). The samples were immediately mixed with 1 μl matrix solution (25 mg/ml 3,5-dimethoxy-4-hydroxycinnamic acid [sinapinic acid; Bruker Daltonics, Bremen, Germany]) in 50% (vol/vol) acetonitrile (Sigma-Aldrich) supplemented with 0.6% (vol/vol) trifluoroacetic acid (Roth, Germany). The matrix sample spots were crystallized by air drying.

Whole-cell MALDI-TOF MS parameters. The MALDI-TOF MS analysis has been described previously (13). Briefly, mass spectra were acquired with an Ultraflex II MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an all-solid-state Smartbeam Nd:YAG laser and operated at 100 Hz in the positive linear mode (delay, 100 ns; voltage, 25 kV; molecular mass range, 2.2 to 40 kDa) under the control of Flexcontrol software (version 3.0; Bruker Daltonics). Each spectrum was obtained by averaging up to 7,000 laser shots acquired at the minimum laser power necessary for ionization of the samples. Automated spectrum acquisition was performed using the Auto Execute software integrated within Flexcontrol.

Data evaluation. Mass data files were transferred to the Flexanalysis software (version 3.0; Bruker Daltonics) and processed with baseline correction, Gaussian smoothing, and peak finding. Average mass values were determined. Spectra were internally calibrated using a set of ribosomal biomarker proteins common to most *Salmonella enterica* subsp. *enterica* strains, including ribosomal proteins RL36 (*m*/*z* 4,365.3), RL32 (*m*/*z* 6,316.2), RL30 (*m*/*z* 6,383.6), RS15 (*m*/*z* 10,067.5), RS19 (*m*/*z* 10,286.1), RL25 (*m*/*z* 10,542.2), RS14 (*m*/*z* 11,478.3), RS10 (*m*/*z* 11,767.6), and DNA-binding protein H-NS (*m*/*z* 15,412.5) (10). A maximum of 300 peaks with a signal-to-noise ratio minimum of 2 were selected in the range of 2,000 to 25,000 Da. For species and subspecies identifications, peak lists were imported into the SARAMIS software (Spectral Archiving and Microbial Identification System, release 3.4; Anagnostec GmbH, Germany). In the first step, a consensus spectrum, called the superspectrum, was calculated based on duplicate spectra of 221 *S. enterica* subsp. *enterica* strains belonging to different serovars. This superspectrum was used for identification of *S. enterica* subsp. *enterica* at the subspecies level and consisted of sets of biomarker ions that were present in >90% of the strains and displayed specificities at the genus, species, and subspecies levels. Clinprotools software (version 2.1; Bruker Daltonics) was used for peak definition (signal to noise ratio of >1), integration (endpoint level), mass recalibration (maximal peak shift of 200 ppm), area normalization (against total ion count). Receiver operating characteristic (ROC) analysis was performed in order to prescreen for serovar-identifying biomarker ions for the most prevalent serovars. ROC curves were generated for each peak as two subgroups (a set of spectra from multiple strains of the "target" serovar and a second subset of spectra from all other serovars) and were compared by using a graphical display of the false-positive rate (horizontal axis) and the true-positive rate (vertical axis). The optimal ROC curve, meaning the highest true-positive rate for a given false-positive rate, corresponds to an area under the ROC curve (AUC) of 1. Potential biomarker candidates were further analyzed by visual inspection of the mass spectra by Flexanalysis (overlaid view). As a general rule, mass signals were preferably selected as potentially serovar specific if they were present in all sets of data obtained for a given serovar and were absent in all other serovars, resulting in an AUC value of near 1. For identification of *Salmonella* strains below species level, weighted pattern-matching approaches were conducted. By using the SARAMIS software, weighted superspectra were created, and highly discriminative biomarkers were upweighted (given an overemphasized value) in the identification routine, while nonspecific, variable, and low-intensity peaks were downweighted or ignored in the identification process. Automated computer-aided identification was performed by comparing peak lists for individual samples with the established reference database of superspectra, generating a ranked list of matching spectra based on the point value system. The established procedures were then used to screen an additional 692 *S. enterica* subsp. *enterica* strains comprising 89 serovars for the presence of serovar-identifying biomarker ions.

Identification of biomarker proteins based on database searches. The *m*/*z* peaks obtained were subjected to an online TagIdent protein database search (18). An unrestricted search with pI values was performed. Theoretical masses of protein sequences were calculated using the PeptideMass tool (18). The BLAST servers at www.sanger.ac.uk and www.expasy.ch were used for protein-versus-translated DNA BLAST searches in 19 *Salmonella* genome completed sequences and 20 *Salmonella* whole-genome available shotgun sequences.

RESULTS

***Salmonella enterica* subsp. *enterica* identification.** Whole-cell MALDI-TOF MS for *S. enterica* subsp. *enterica* (subspecies) identification, which is a prerequisite for serovar identification, was performed with 221 *Salmonella* strains essentially as described in reference 13. As a major *Salmonella enterica* subsp. *enterica*-specific biomarker ion, a mass peak at *m*/*z* 14,395, corresponding to the ribosomal protein L17, was used and given an overemphasized value in the superspectrum. This peak was present in almost all 89 different *S. enterica* subsp. *enterica* serovars tested (except serovars Gallinarum var. Pullorum and Schleissheim) but absent in all other *S. enterica* subspecies and *S. bongori*. Non-*S. enterica* subsp. *enterica* and *S. bongori* strains, on the other hand, were characterized by a mass peak at *m*/*z* 14,453, corresponding to ribosomal protein L17 with an additional serine residue and a threonine-to-alanine mutation.

Identification of potential serovar-identifying biomarker ions. Discrimination ability at the serovar level was determined by analyzing data subsets comprised of multiple MALDI-TOF MS spectra obtained from different serovars. Typically, when two different serovars were compared, not more than 15 potentially serovar-discriminating peaks per spectrum were observed, of which usually several were present in more than one serovar. Therefore, the spectra were carefully inspected for reproducible serovar-identifying biomarkers and combinations thereof. Promising markers displaying high AUC values (near to 1) were selected accordingly and evaluated for their usage to discriminate either single serovars or groups of serovars (Table 2). Table 3 shows additional mass peaks displaying characteristic mass shifts in subsets of strains belonging to different serovars that assisted serovar identification based on major serovar-identifying signals. Based on sequence data from completed genome sequencing or whole-shotgun genome sequencing of more than 24 *Salmonella* strains belonging to different serovars, the detected mass signals that could be assigned to

TABLE 1. *Salmonella enterica* subsp. *enterica* strains (n = 913) used in this study

Serovar	No. of strains	Source(s)	Yr(s) isolated
Enteritidis	53	Poultry, bovine, swine, food, environmental	2003–2008, 2010
Typhimurium/4,[5],12:i:-	101/27	Bovine, poultry, swine, food, others	2000–2010
Infantis	51	Bovine, poultry, swine, human, food, environmental	2000–2010
Virchow	32	Poultry, swine, human, cat, food, environmental	2000–2001, 2003–2009
Hadar	52	Bovine, poultry, human, cat, dog, bird, food	2001–2009
Bovismorbificans	14	Poultry, swine, bovine, food	2002–2008
Derby	74	Poultry, swine, human, dog, environmental, food	2001–2010
Anatum	17	Poultry, swine, bovine, food	2002–2008
Newport	48	Poultry, bovine, human, dog, fish, food, environmental	2000, 2002–2010
Goldcoast	24	Swine, bovine, rabbit, environmental, food	2001–2006, 2008–2009
4,12:d:-	13	Poultry, swine, bovine	2003–2004, 2008
Abony	6	Bovine, horse	2007–2008
Agama	1	Swine	2007
Agona	7	Swine, dog, poultry, food, environmental	2005, 2008
Albany	1	Fertilizer	2006
Amersfoort	1	Swine	2005
Augustenborg	1	Poultry	2002
Bareilly	2	Environmental, food	2006
Blockley	11	Poultry, bovine, swine	2000, 2005–2009
Braenderup	4	Swine, food, mussel	2005–2007
Brandenburg	9	Swine, poultry, bovine, human, fertilizer	2002, 2005–2007, 2010
Bredeney	10	Poultry, environmental, dog, bird, food	2005, 2008, 2010
Chandans	1	Food	2010
Cerro	1	Chicken	2008
Choleraesuis	5	Swine, game, cat	2002, 2004–2006, 2009
Coeln	2	Chicken	2008
Colindale	1	Food	2003
Corvallis	2	Animal food	2006
Cubana	6	Poultry, animal food, fertilizer	2006, 2008–2009
Dublin	11	Swine, bovine, poultry, dog, food	2005–2008
Ealing	5	Dog, animal food, environmental	2002–2004, 2006
Eastbourne	4	Food, poultry	2005, 2009
Emek	1	Sludge	2007
Falkensee	2	Animal food	2004–2005
Ferruch	1	Poultry	2002
Gallinarum/Gallinarum var. Pullorum	4/10	Poultry	2004–2010
Give	4	Swine, poultry, food	2006, 2008
Glostrup	4	Swine, environmental, food	2000, 2006, 2008
Haardt	3	Poultry	2000–2001
Haifa	5	Swine, human, fertilizer, food	2005–2006, 2008
Heidelberg	8	Poultry, rabbit	2003, 2005, 2007–2008
Indiana	8	Poultry, environmental	2006, 2008
Isangi	4	Poultry	2006, 2008–2009
Israel	1	Deer	1998
Javiana	4	Food, dog, swine, environmental	2004, 2006–2008
Jerusalem	1	Poultry	2008
Kentucky	3	Swine, human, food	2005, 2007–2008
Kiambu	11	Cat, poultry, dog, environmental	2000, 2004–2006, 2008–2009
Kottbus	16	Poultry, bird, bovine, swine, environmental, sludge	2000, 2002–2006, 2008
Lagos	1	Not known	1983
Lexington	5	Swine, poultry, food	2006–2008
Litchfield	5	Swine, poultry, environmental	2000–2001, 2005–2006
Livingstone	17	Poultry, swine, food, dog	2006–2008, 2010
London	16	Swine, bovine, poultry, food	2000–2009
Manchester	2	Poultry	2002, 2005
Manhattan	12	Poultry, bovine, swine	2000–2001, 2004–2009
Mbandaka	9	Food, poultry, swine, environmental	2001, 2006–2010
Meleagridis	1	Human	2009
Minnesota	1	Not known	2010
Molade	1	Food	2009
Montevideo	4	Poultry	2005, 2007, 2010
Muenchen	5	Bovine, dog, game	2004, 2006, 2008–2009
Muenster	3	Poultry, swine, food	2005, 2010
Ohio	4	Poultry, food	2006, 2008
Oranienburg	2	Sludge, environmental	2006, 2010
Orion	12	Poultry, bovine, fish, environmental, food, sludge	2000–2001, 2005–2007, 2009
Pakistan	1	Poultry	2000
Panama	12	Swine, poultry, food	2000, 2003–2004, 2006–2008, 2010
Paratyphi A	1	Human	1999
Paratyphi B	12	Poultry, bovine	2003, 2004, 2008, 2010
Poona	1	Food	2008
Reading	6	Food, poultry, dog	2001, 2003–2005, 2007
Rissen	5	Poultry, bovine, swine, food	2004, 2008

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TABLE 1—*Continued*

Serovar	No. of strains	Source(s)	Yr(s) isolated
Saintpaul	20	Poultry, swine	2003, 2006, 2008, 2010
Sandiego	7	Dog, mouse, bird, fertilizer, food	2005–2007, 2009–2010
Schleissheim	7	Dog, bird, game, environmental	2002, 2006, 2007
Schwarzengrund	4	Swine, poultry, food, fertilizer	2006, 2008
Senftenberg	13	Poultry, swine, food, environmental	2001, 2003, 2005, 2008–2010
Stanley	8	Swine, dog, food	2004–2005, 2007–2008
Stanleyville	8	Poultry, bovine, food wastewater	1998, 2001, 2007, 2009
Stourbridge	1	Badger	2010
Teddington	1	Food	2010
Tennessee	3	Food, fertilizer	2006, 2008
Thompson	4	Swine, poultry, bovine	2005, 2007–2008
Weltevreden	4	Food, bovine, seafood	2005, 2008
Wien	3	Dog, environmental, food	2004, 2006–2007
Wilhelmsburg	1	Duck	1998
Zanzibar	4	Fish, wild pig	2000, 2006–2007

known proteins and possible point mutations explaining the detected mass shifts were identified. While mass spectrometric species identification mainly relies on sets of ribosomal proteins, many of the amino acid polymorphisms that resulted in potentially serovar-identifying mass variations were found in other protein classes, including many proteins with unknown functions. For example, a mass signal at *m/z* 6,036 was uniquely found in serovar Enteritidis and therefore useful for identifying this serovar. On the other hand, the presence of a mass signal of the same protein at *m/z* 6,009 indicated another serovar. Based on genome database searches, the corresponding mass difference of 27 Da could be explained by an S→N exchange in a putative uncharacterized protein (Fig. 1). Similarily, Leuschner et al. (33) found a mass signal at *m/z* 6,030 as one peak that was present in all of the five serovar Enteritidis strains they analyzed. A mass signal at *m/z* 7,097 of a putative uncharacterized protein, YaiA, was selected to differentiate serovar Typhimurium or its related monophasic variant (15, 57) from almost all other serovars, which mostly displayed a peak of the corresponding protein at 7,111 (K→N). The same peak was also found in serovar Virchow and one subtype of serovar Newport (Newport-II), but those could be differentiated from serovar Typhimurium by using additional markers. Serovar Virchow could be clearly identified by the presence of a mass peak at *m/z* 10,048 instead of *m/z* 10,067, corresponding to an R→H substitution in ribosomal protein S15. Serovar Infantis (6,7,14:r:1,5) and the related but rarely isolated serovars Augustenborg (6,7,14:i:1,2) and Colindale (6,7:r:1,7) showed a characteristic mass shift in the putative uncharacterized protein YciF at *m/z* 18,635 (Fig. 1). Serovars Augustenborg and Colindale in turn could be differentiated from serovar Infantis, e.g., by using the protein Gns as an assisting biomarker at *m/z* 6,484, which displayed a mass signal at *m/z* 6,512 in serovar Infantis (Table 2). Serovar Hadar was characterized by the presence of a mass peak at *m/z* 10,927 (protein YbgS), which was also found in strains of serovars Blockley, Ealing, Ferruch, Haardt, Kiambu, Kottbus, Orion, and 4,12:d:-. By using assisting peaks at *m/z* 6,484 (Gns), 6,771 (YhfG), 8,699 (putative inner membrane protein), 9,404 (unidentified), 10,542 (RL25), 17,461 (RS7), and 18,655 (YciF), serovar Hadar could be differentiated from all other serovars tested. Notably, host-restricted or host-adapted serovars, like serovar Choleraesuis (swine), serovar Paratyphi A (human), and serovar Gallinarum/Pullorum (chicken), showed more mass variations than other serovars and could be readily identified by several serovar-identifying biomarkers, even allowing subtyping between *Salmonella enterica* serovar Gallinarum biotype Gallinarum and biotype Pullorum. These nonmotile strains cannot be distinguished by serological methods and have to be subtyped on the basis of biochemical characteristics. Using MALDI-TOF MS, biotype Pullorum could readily be differentiated from biotype Gallinarum on the basis of at least 9 mass variations caused by amino acid polymorphisms in several proteins (Table 4). Moreover, biotype Pullorum was the only serovar that displayed a serovar-specific deficiency in post-translational modification of, e.g., ribosomal proteins RS11 (*m/z* 13,701 instead of 13,715; methylation), RS12 (*m/z* 13,607 instead of 13,653; β-methylthiolation), RL33 (*m/z* 6241 instead of 6255; methylation), and RL16 (*m/z* 15,194 instead of 15,225; unknown modification). Similarly, potential serovar-specific mass signals were detectable for serovars Manhattan, Heidelberg, London, Tennessee, Schleissheim, and 4,12:d:- (Table 2).

Some serovars showed a notable degree of intraserovar variability in their MALDI-TOF profiles. For example, serovar Derby showed at least two MALDI-TOF subtypes, designated Derby-I and Derby-II, of which one could be identified using peaks at *m/z* 6,302 (instead of 6,316 for ribosomal protein L32) and *m/z* 7,983 (instead of 7,993 for protein YibT). Serovar Newport could be also divided in different subtypes, one of which was characterized by mass signals at *m/z* 5,966 and 17,474 (RS7) (mainly avian strains), whereas another contained corresponding signals at *m/z* 6,009 and 17,461 (Newport-I and Newport-III). Whole-cell MALDI-TOF MS subtypes have been also identified in serovar Paratyphi B D-tartrate positive and Ealing. Serovars Newport, Paratyphi B, and Derby have been already described to be polyphyletic according to both multilocus enzyme electrophoresis (MLEE) and multilocus sequence typing (MLST) studies, while most other *Salmonella* serovars represent monophyletic lineages (6, 42, 47). Apparently, MALDI-TOF MS subtyping, similar to MLST, reflects different evolutionary lineages for polyphyletic serovars, in contrast to serotyping.

Validation for identification of five frequently isolated *Salmonella* serovars. To validate the approach for the identification of the five frequently isolated serovars Enteritidis, Typhimurium, Virchow, Hadar, and Infantis in humans in Europe,

TABLE 2. Selected biomarkers for serovar-level identification of single serovars or groups of serovars (major serovar-identifying peaks)

Exptl mass (avg *m/z*)	Tentative protein identity	Predicted mass (Da)	Serovar(s) in which biomarker found	Antigenic formula	Mass in other serovars (avg *m/z*)	Predicted mass (Da)	Amino acid exchange
5,966	Probably putative uncharacterized protein	NA[a]	Newport-I	6,8:e,h:1,2	6,009	6,009.3[b]	NA
6,022	Putative uncharacterized protein YciG	6,022.3[a]	Choleraesuis	6,7:c:1,5	6,094	6,094.4	E→G
6,036	Putative uncharacterized protein	6,036.3[b]	Enteritidis	1,9,12:g,m:-	6,009	6,009.3[b]	S→N
6,512	Gns	6,511.6	Newport-I	6,8:e,h:1,2	6,484	6,483.5	A→V
			Infantis	6,7,14:r:1,5			
			Manhattan	6,8:d:1,5			
			Mbandaka	6,7,14:z_{10}:e,n,z_{15}			
			4,12:d:-	4,12:d:-			
			Paratyphi A	1,2,12:a:-			
			Stanleyville	1,4,12:$z_{4,}$ z_{23}:(1,2)			
			Zanzibar	3,(10),(15):k:1,5			
			Lexington	3,10:z_{10}:1,5			
6,587	Ribosome modulation factor	6,586.6	Paratyphi A	1,2,12:a:-	6,572	6,572.5	D→E
7,097	Putative uncharacterized protein YaiA	7,096.8[b]	Typhimurium/4,5,12:i:-	4,5,12:i:(1,2)	7,111	7,110.9[b]	K→N
			Virchow	6,7,14:r:1,2			
			Newport-II	6,8:e,h:1,2			
7,139	Putative uncharacterized protein YaiA	7,138.9[b]	Heidelberg	1,4,[5],12:r:1,2	7,111	7,110.9[b]	A→V
7,983	Uncharacterized protein YibT	NA	Derby-I	1,4,[5],12:f,g:[1,2]	7,993	7,993.1	NA
8,023	Uncharacterized protein YibT	8,023.1[b]	Heidelberg	1,4,[5],12:r:1,2	7,993	7,993.1	G→S
		NA	Thompson	6,7,14:k:1,5			NA
			Blockley	6,8:k:1,5			
			Haardt	8:k:1,5			
8,027	Uncharacterized protein YibT	8,027.1[a]	Tennessee	6,7,14:z_{29}:[1,2,7]	7,993	7,993.1	L→F
8,061	Probably putative cytoplasmic protein YecF	NA	4,12:d:-	4,12:d:-	8,051	8,051.2[b]	NA
8,151	RNA binding translation initiation factor IF-1	8,151.5[b]	Paratyphi A	1,2,12:a:-	8,119	8,119.4	V→M
8,342	Putative stress response protein YjbJ	8,342.3[b]	Tennessee	6,7,14:z_{29}:[1,2,7]	8,329	8,329.3[b]	T→N
9,548	Probably DNA-binding protein HU-α	NA	Manhattan	6,8:d:1,5	9,523	9,521.9	NA
10,048	Ribosomal protein S15	10,048.5[b]	Virchow	6,7,14:r:1,2	10,067	10,067.5[b]	R→H
10,381	Probably ribosomal protein L17	NA	Schleissheim	4,12,27:b:-	14,395	14,395.6	NA[c]
			Gallinarum var. Pullorum	1,9,12:-:-			
10,528	Ribosomal protein L25	10,528.2	Manhattan	6,8:d:1,5	10,542	10,542.2	I→V
			Braenderup	6,7:e,h:enz$_{15}$			
			Newport-III	6,8:e,h:1,2			
			Haifa	1,4,[5],12:z_{10}:1,2			
			Stanley	1,4,[5],12:z_{10}:1,2			
				4,5,12,27:d:1,2			
10,569	Probably RL25	NA	Schleissheim	4,12,27:b:-	10,542	10,542.2	NA
10,872	Probable σ^{54} modulation protein	10,872.4	Heidelberg	1,4,[5],12:r:1,2	10,858	10,858.4	V→I
10,886	Probable σ^{54} modulation protein	10,886.4	Cholerasuis	6,7:c:1,5	10,957	10,958.5[c]	K→R
10,899	Uncharacterized protein YbgS	10,900.5[c]	Manhattan	6,8:d:1,5	10,957	10,958.5[c]	D→G
			Choleraesuis	6,7:c:1,5			
			Muenchen	6,8:d:1,2			
			Haifa	1,4,[5],12:z_{10}:1,2			
10,927	Uncharacterized protein YbgS	10,928.5[c]	Hadar	6,8:z_{10}:e,n,x	10,957	10,958.5[c]	T→A
			Kottbus	6,8:e,h:1,5			
			Haardt	8:k:1,5			
			Blockley	6,8:k:1,5			
			Ferruch	8:e,h:1,5			
			Kiambu	1,4,12:z:1,5			
			4,12:d:-	4,12:d:-			
			Orion	3,10:y:1,5			
			Ealing-I	35:g,m,s:-			

10,947	Probably uncharacterized protein YbgS	NA	Brandenburg	4,[5],12:l, v:enz$_{15}$	10,957	10,958.5[c]	NA
			Panama	1,9,12:l,v:1,5			
			Israel	9,12:e,h:enz$_{15}$			
			Reading	4,12:e,h:1,5			
			Sandiego	1,4,[5],12:e,h:enz$_{15}$			
			Eastbourne	1,9,12:e,h:1,5			
10,969	Probably uncharacterized protein YbgS	NA	Aba	6,8:i:enz$_{15}$	10,957	10,958.5[c]	NA
			Sandiego	1,4,[5],12:e,h:enz$_{15}$			
10,988	Uncharacterized protein YbgS	10,988.5[c]	Heidelberg	1,4,[5],12:r:1,2	10,957	10,958.5[c]	G→S
11,591	Ribosomal protein L21	11,591.8	Paratyphi A	1,2,12:a:-	11,579	11,578.4	V→I
14,381	Ribosomal protein RL17	NA	Schleissheim	4,12,27:b:-	NA	14,395.6	NA
14,981	Ribosomal protein RL15	NA	London	3,(10)(15):l,v:1,6	14,967	14,967.4	NA
15,342	Probably DNA binding protein H-NS	NA	Paratyphi B-I	1,4,[5],12:b:1,2	15,412	15,412.5[b]	NA
15,471	DNA binding protein H-NS	15,470.5[b]	Paratyphi A	1,2,12:a:-	15,412	15,412.5[b]	G→D
17,432	Ribosomal protein RS7	17,432.1[b]	Choleraesuis	6,7:c:1,5	17,461/474		V→A
18,635	Putative uncharacterized protein YciF	18,634.2	Infantis	6,7,14:r:1,5	18,644/655	18,643.7	R→H/P→S
			Augustenborg	6,7,14:i:1,2			
			Colindale	6,7:r:1,7			

[a] NA, no sequence data available.
[b] Methionine loss.
[c] Signal peptide cleavage.

692 various *Salmonella* strains comprising 89 serovars were analyzed by automated MALDI-TOF MS analyses. The results were compared with traditional serotyping results. The selectivity for the top 5 serovars of the newly developed method is shown in Table 5, including the major discriminative serovar-identifying biomarker ions. Exclusivity was 100% for all five serovars, meaning that none of the nontarget samples was false positively identified. Inclusivity was 100% for serovars Typhimurium, Virchow, and Infantis. In cases of serovars Enteritidis and Hadar, a reduced inclusivity was observed (86.5% and 85.7%), resulting in a reduced selectivity of the method. While 32 strains were correctly identified as serovar Enteritidis (true positives), 5 strains lacked the diagnostic marker ion for serovar Enteritidis at *m/z* 6,036. Those strains lacked a detectable expression of a set of about 10 to 15 proteins, including some of the diagnostic marker peaks used in this study. This set of proteins included, besides several uncharacterized proteins with unknown functions, the putative homeobox protein YbgS and the hydrophilins YciG, YciF, and YciE and YjbJ, which are known to be highly abundant under certain stress conditions (Table 6). Expression levels of proteins listed in Table 6 varied strongly according to peak intensities observed in whole-cell MALDI-TOF MS. However, when fresh isolates from routine diagnostics were analyzed, absence of these peaks was rarely observed, keeping the number of false negatives in routine diagnostics to a minimum. In addition, it was reproducibly observed that expression of this set of proteins was not detected or barely detectable in some strains when bacterial biomass was taken from single colonies instead of bacterial smears taken from the same agar plate. When bacteria were grown as biofilm-like smears, these proteins usually were more highly expressed, and therefore both sample preparation procedures were routinely performed for MALDI-TOF MS-based *Salmonella* serovar screening.

To assess the repeatability of the method, selected strains of each top 5 serovar were tested in series of eight replicates. Furthermore, five strains of each top 5 serovar were reanalyzed after a period of 2 years under the same conditions. The presence and absence of the serovar-identifying biomarker ions have been confirmed in all spectra, and all strains have been reidentified on the serovar level (data not shown).

DISCUSSION

The aim of this study was primarily to evaluate whole-cell MALDI-TOF mass spectrometry as a prescreening tool for rapid identification of epidemiologically important serovars, with emphasis on five frequently isolated *Salmonella* subsp. *enterica* serovars in Europe, namely, Enteritidis, Typhimurium, Infantis, Virchow, and Hadar, thereby reducing numbers of samples that have to be analyzed by traditional serotyping in combination with biochemical test reactions.

For identification of specific serovars, a hierarchical decision tree network-like approach was developed and initially validated (Fig. 2). It consists of a sequential scanning of mass spectra for the presence or absence of peaks displaying specificities at different taxonomic levels, namely, sets of genus-, species-, subspecies-, or serovar-identifying biomarkers. A future computer-aided identification scheme could be based on such a hierarchical approach, applying suitable algorithms for

TABLE 3. Mass peaks frequently displaying mass shifts detected by whole-cell MALDI-TOF MS of *Salmonella enterica* subsp. *enterica* serovars[a]

Tentative protein identity	Observed mass (Da)	Predicted mass (Da)	Amino acid substitution(s) compared to serovar Typhimurium sequence	Example(s) of prevalent serovars containing signal
Uncharacterized protein YhfG	6,799	6,799.8 $(M+H)^+$		Typhimurium, Virchow
	6,771	6,771.8 $(M+H)^+$	D→S	Enteritidis, Hadar
	6,718	6,718.7 $(M+H)^+$	R→C/V→A	Infantis
	6,757	NA	NA	Goldcoast
NA	8,418	NA	NA	Typhimurium, Virchow, Infantis,
	8,445	NA	NA	Hadar
Putative inner membrane protein STM0348	8,686	8,687.3 $(M+H)^+$		Typhimurium, Enteritidis
	8,699	8,699.3 $(M+H)^+$	M→L/A→T	Virchow, Infantis, Hadar
NA	9,404			Typhimurium, Virchow, Hadar
	9,390			Enteritidis
	9,374			Infantis
Ribosomal protein RL21	11,565	11,565.4 $(M+H)^+$	I→V	
	11,579	11,578.4 $(M+H)^+$		Typhimurium, Enteritidis, Virchow, Hadar, Infantis
Putative inner membrane protein YgaM	11,848	11,848.4 $(M+H)^+$		Typhimurium, Virchow, Hadar
	11,857	11,857.5 $(M+H)^+$	Q→H	Enteritidis, Infantis
Ribosomal protein RS7	17,461	17,460.2 $(M-M+H)^+$		Typhimurium, Virchow, Infantis, Hadar
	17,474	17,474.2 $(M-M+H)^+$	D→E	
Putative uncharacterized protein YciF	18,654	18,653.2 $(M+H)^+$		Typhimurium, Virchow, Hadar
	18,644	18,643.2 $(M+H)^+$	P→S	Enteritidis
	18,635	18,634.2 $(M+H)^+$	R→H	Infantis
Superoxide dismutase (Mn)	22,949	22,948.9 $(M-M+H)^+$		Typhimurium
	23,012	23,010.9 $(M-M+H)^+$	S→D/L→F	Enteritidis
	29,979	22,976.9 $(M-M+H)^+$	S→D/N	Infantis, Virchow, Hadar

[a] NA, not assigned.

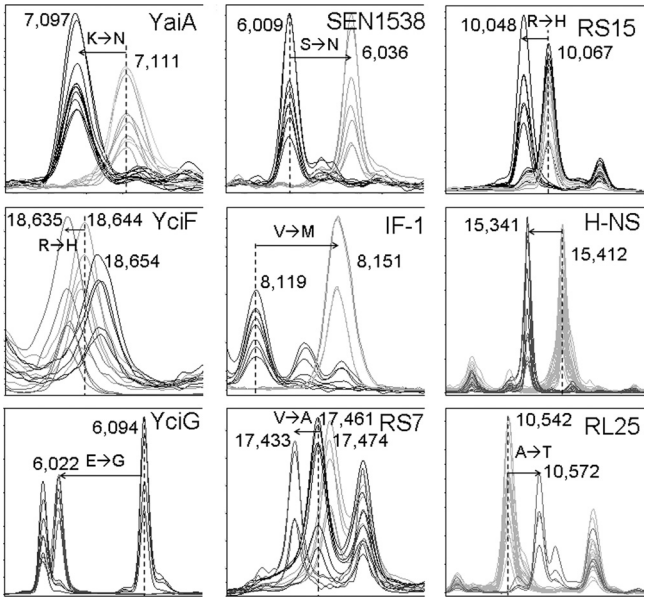

FIG. 1. Examples of serovar-identifying biomarker ions found in MALDI-TOF MS spectra of *S. enterica* subsp. *enterica*. For specificities and details, see Tables 2 and 3.

different taxonomic levels in a sequential mode. Generally, when higher taxonomic resolution is required, as with serovar level identification, a pattern recognition approach would be of limited use, because of the complexity of the peak patterns.

TABLE 4. Differentiation of serovars Gallinarum and Gallinarum biotype Pullorum based on specific sets of serovar-identifying biomarker ions

Tentative protein identity	Observed mass (Da)		Amino acid substitution or posttranslational modification
	Gallinarum biotype Pullorum	Gallinarum	
RL33	6,241	6,255	+ Methyl
YibT	7,993	7,965	K→T
YecF	8,051	8,038	T→S
RS20	9,523	9,553	A→V
RL25	10,542	10,572	A→T
RP5 M	10,830 (only subtype)	10,858	NA[a]
RL7/RL12	ND[b]	12,170	NA
Unknown	12,211	12,211	NA
RL18	12,771	12,776	NA
RS12	13,606	13,652	+ β-Methylthiolation
RS11	13,701	13,715	+ Methyl
RL17	14,382	14,395	NA
RL16	15,194	15,225	Unknown
Unknown	16,306	16,306	NA
Superoxide dismutase	23,011	23,045	L→F

[a] NA, no sequence data available.
[b] ND, not determined.

TABLE 5. Major serovar-identifying peaks used for MALDI-TOF screening of the top 5 serovars and results of selectivity validation[a]

Parameter	Enteritidis	Typhimurium	Virchow	Infantis	Hadar
Antigenic formula	1,9,12:g,m:-	1,4,[5],12:i:1,2/1,4,[5],12:i:-	6,7,14:r:1,2	6,7,14:r:1,5	6,8:z10:e,n,x
Selectivity (%)	99.3	100	100	100	99.1
Exclusivity (%)	100	100	100	100	100
Inclusivity (%)	86.5	100	100	100	85.7
m/z					
Putative uncharacterized protein	**6,036**	6,009	**6,008**	6,009	6,009
Protein Gns	6,484	6,484	6,484	**6,512**	6,484
Uncharacterized protein YhfG	6,771	6,799	6,799	6,718	6,771
Putative uncharacterized protein YaiA	7,111	**7,097**	**7,097**	7,111	7,111
Putative inner membrane protein STM0348	8,686	**8,686**	8,699	8,699	**8,699**
NA[a]	9,390	9,404	9,404	9,374	**9,404**
Ribosomal protein RS15	10,067	**10,067**	**10,048**	10,067	10,067
Uncharacterized protein YbgS	10,958	10,958	10,958	10,958	**10,927**
Putative inner membrane protein YgaM	11,857	11,848	11,848	11,857	11,848
Ribosomal protein RS7	17,474	17,461	17,461	17,461	17,461
Putative uncharacterized protein YciF	18,644	18,655	18,655	**18,635**	**18,654**
Superoxide dismutase SodM (Mn)	**23,012**	**22,949**	22,979	22,979	22,979

[a] Highly specific biomarker combinations are indicated in boldface.

Weighted pattern matching approaches could be established using average spectra (superspectra, main spectra) that contain a limited number of selected highly serovar-discriminative peaks, which are given high discriminative values (upweighted peaks) in the identification routines. Variable expression rates of biomarker proteins due to differential regulation, especially of nonhousekeeping proteins, might be observed, possibly leading to false negatives when the biomarker concentration is below the detection limit. As in the case of serovar Enteritidis, some strains reproducibly lacked expression of a subset of proteins under different conditions. Consequently, the obligatory presence of those biomarkers can be regarded as internal control mass peaks. It is interesting that those affected proteins are very likely to be controlled by the global regulator RpoS, which is known to control expression of proteins involved in stress response and virulence (17, 52). Under certain condi-

TABLE 6. Major highly upregulated (differentially expressed) proteins (potentially *rpoS* dependent)

Tentative protein identity	Observed mass(es) (Da)	Function/regulation	Reference(s)[a]
Putative cytoplasmic protein (STM1513)	6,009, 6,036	YciG paralogue, upregulated in 2-day-old minimal medium, hydrophilin	40
Putative uncharacterized protein YciG (Q7CQG0_SALTY)	6,094	Cotranscribed with YciF, induced in stationary phase, hydrophilin	40, 53
Putative uncharacterized protein YhfG (YHFG_SALTY)	6,799, 6,771, 6,718	Unknown/stationary-phase protein, biofilm-specific pattern of expression	53
Putative uncharacterized protein YaiA (Q8ZRE9_SALTY)	7,097, 7,111	Peroxide induction	53
Putative periplasmic or secreted protein YahO (Q7CR49_SALTY)	7,661	Unknown	25, 38
Uncharacterized protein YibT (YIBT_SALTY)	7,993	Unknown	
Putative uncharacterized protein YjbJ (YJBJ_SALTY)	8,329	Highly abundant, very high RpoS dependence in stationary phase, induced by osmotic stress, dispensable in *E. coli* grown in rich or minimal medium, hydrophilin	50, 53
Putative inner membrane protein (Q8ZRH2_SALTY)	8,686, 8,699	Putative inner membrane protein	
Putative homeobox/exported protein YbgS (YBGS_SALTY)	10,927, 10,956	Function unknown, upregulated in 2-day-old minimal medium, upregulated in biofilm	38, 53
Inner membrane protein YgaM (Q8ZML4_SALTY)	11,848, 11,857	Stress induced, upregulated in biofilm	53
Putative LysM domain protein YgaU (Q8ZML9_SALTY)	15,991	Cell wall catabolic process	25, 50, 53
Putative uncharacterized protein YciF (Q7CQF9_SALTY)	18,644, 18,655	Upregulated in 2-day-old minimal medium, induced by osmotic stress imposed by NaCl, unique to biofilm, putative structural protein, osmotically induced, belongs to the σ^S regulon	25, 40, 53
Putative cytoplasmic protein YciE (Q7CQF8_SALTY)	18,974	Induced by acid shock	40

[a] Reference(s) describing RpoS dependence under one or more environmental conditions in *E. coli* or *Salmonella* for the given protein.

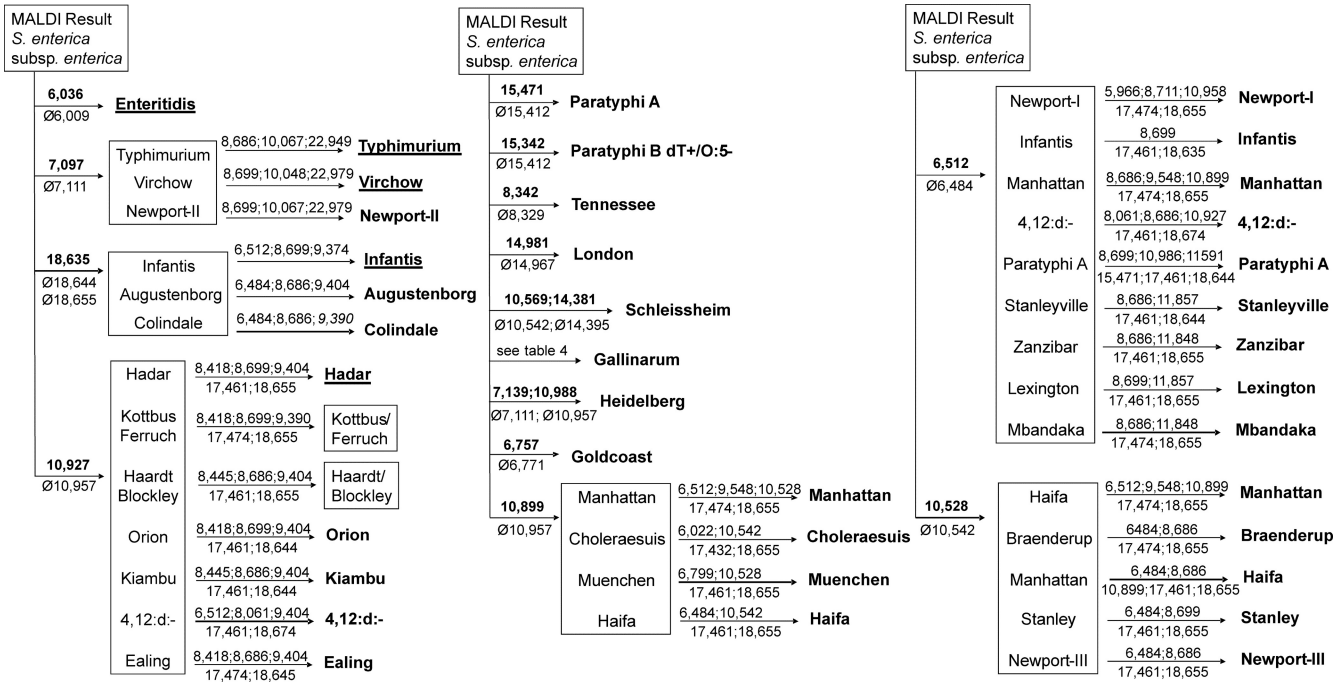

FIG. 2. Decision tree classification for identification of selected *Salmonella enterica* subsp. *enterica* serovars using MALDI-TOF MS. Starting from a MALDI-TOF MS-based identification result (here, at the subspecies level), spectra are screened for the absence/presence of a limited number of serovar-identifying mass peaks. Major serovar-identifying biomarker ions are indicated in bold. Ions absent from respective serovars are indicated by Ø followed by the *m*/*z* value. Selected ions useful for further discrimination within groups of serovars (framed) are shown above and below the right set of arrows. Underlined serovars were validated with an extended reference strain collection (Table 5). For detailed information, see Tables 2 and 3.

tions, mutations within *rpoS* appear to result in a growth advantage in *Escherichia coli* and *Salmonella*, leading to a selection of *rpoS* mutants in bacterial populations (36). Since RpoS frequently is a target for point mutations modifying bacterial fitness, it can be assumed that some of the strains acquired point mutations during passage and storage that led to inhibition of RpoS-regulated proteins. Such strains typically displayed wrinkled colony morphology when grown on agar plates. It has already been reported that cultures of virulent *S. enterica* serovar Enteritidis can convert from the smooth to a new wrinkled (lacy) colonial phenotype without loss of antigen after storage in nutrient agar stabs for longer periods (20). RpoS mutants were also frequently found from highly passaged laboratory strains of *Salmonella* (39).

This method could be a valuable tool when integrated into microbiological routine diagnostics, because prevalent serovars could easily be identified at low costs, while the number of isolates that would have to be subsequently identified by traditional and more laborious methods would be significantly reduced. Several studies have calculated the cost of MALDI-TOF mass spectrometry when used for typing of organisms (7, 10, 11, 44). Generally, they concluded that the technique is rather cost-efficient and rapid compared to other typing methods. We estimated that the average cost for serovar identification using MALDI-TOF MS of a strain is between $6.00 and $10.00, including consumables and personnel costs. In our calculation, this price is at least 3-fold lower than the one for serotyping and biochemical test reactions. However, laboratories need to purchase a MALDI-TOF MS instrument, but the expense of such an instrument is meanwhile comparable to other common laboratory equipment needed for molecular bacterial typing (e.g., sequencers or apparatus for pulsed-field gel electrophoresis [PFGE]). Furthermore, the higher the throughput rate of samples in a laboratory, the lower the costs of the analysis per strain. Sample preparation and duration of analysis times are rather rapid. On average, with the strain cultured as a single colony on an agar plate, MALDI analysis requires only a few minutes, compared to hours or days for other molecular typing methods, such as PFGE.

Whole-cell MALDI-TOF MS generates numerical data (mass peaks). Those data are easy to exchange between laboratories, compared to molecular fingerprinting methods, such as PFGE, which is currently accepted as the gold standard method for bacterial typing (5). Therefore, it can be compared with DNA sequence data handling used, for example, in MLST. However, whole-cell MALDI-TOF MS assesses the allelic variation in multiple genes (mainly housekeeping genes) in a strain by determining variations on protein level, in this case, mass variations. Silent mutations are therefore not assessed and, due to the fact that such variations accumulate very slowly in housekeeping genes, the discriminative power might be generally lower than for MLST.

Nevertheless, apparently MALDI-TOF MS subtyping, as described here, similar to MLST reflects different evolutionary lineages for polyphyletic serovars such as Newport, Paratyphi B, or Derby, in contrast to serotyping, while most other *Salmonella* serovars represent monophyletic lineages (21, 42, 47). In a future study the potential of bacterial MALDI-TOF MS

subtyping compared to DNA-based typing methods needs to be elucidated in more detail.

Since identification is not based on the direct detection of antigenic determinants but on surrogate marker proteins, this opens the possibility to assign rough strains to their respective serotypes by using the MALDI-TOF MS approach. Preliminary experiments in our laboratory indicated the usefulness of the method to allocate rough strains derived from serovars Enteritidis and Typhimurium. Suggesting that approximately 3% of *Salmonella enterica* subsp. *enterica* strains received for routine diagnosis in our laboratory are serotyped as rough, MALDI-TOF MS provides a promising advantage in sensitivity compared to serotyping.

In conclusion, the mass spectrometric approach presented here may complement traditional approaches, e.g., as a tool for rapid prescreening of isolates, but the subsequent identification of the majority of serovars will still rely on traditional serotyping. MALDI-TOF MS can function as a prescreening method for *Salmonella* serovar identification. The identification relies rather on certain specific biomarker identification than on pattern recognition. High selectivity was exemplarily shown for five important serovars. Further studies should focus on other frequently isolated serovars.

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