Identification of Highly Pathogenic Microorganisms by Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry: Results of an Interlaboratory Ring Trial

Peter Lasch,^a Tara Wahab,^b Sandra Weil,^c Bernadett Pályi,^d Herbert Tomaso,^e Sabine Zange,^f Beathe Kiland Granerud,^g Michal Drevinek,^h Branko Kokotovic,ⁱ Matthias Wittwer,^j Valentin Pflüger,^k Antonino Di Caro,^l Maren Stämmler,^a Roland Grunow,^m Daniela Jacob^m

Robert Koch Institute, Proteomics and Spectroscopy (ZBS 6), Berlin, Germany^a; Public Health Agency of Sweden, Solna, Sweden^b; Austrian Agency for Health and Food Safety, Vienna, Austria^c; National Center for Epidemiology, Department of Bacteriology, Budapest, Hungary^d; Friedrich-Loeffler-Institut, Institute of Bacterial Infections and Zoonoses, Jena, Germany^e; Bundeswehr Institute of Microbiology, Munich, Germany^f; Norwegian Institute of Public Health, Oslo, Norway^g; National Institute for Nuclear, Chemical and Biological Protection, Milin, Czech Republic^h; National Veterinary Institute, Technical University of Denmark, Frederiksberg, Denmarkⁱ; Spiez Laboratory, Federal Office for Civil Protection, Spiez, Switzerland^j; MABRITEC AG, Riehen, Switzerland^k; Microbiology Laboratory and Infectious Diseases Biorepository, L. Spallanzani National Institute for Infectious Diseases, Rome, Italy^l; Robert Koch Institute, Highly Pathogenic Microorganisms (ZBS 2), Berlin, Germany^m

In the case of a release of highly pathogenic bacteria (HPB), there is an urgent need for rapid, accurate, and reliable diagnostics. MALDI-TOF mass spectrometry is a rapid, accurate, and relatively inexpensive technique that is becoming increasingly important in microbiological diagnostics to complement classical microbiology, PCR, and genotyping of HPB. In the present study, the results of a joint exercise with 11 partner institutions from nine European countries are presented. In this exercise, 10 distinct microbial samples, among them five HPB, *Bacillus anthracis*, *Brucella canis*, *Burkholderia mallei*, *Burkholderia pseudomallei*, and *Yersinia pestis*, were characterized under blinded conditions. Microbial strains were inactivated by high-dose gamma irradiation before shipment. Preparatory investigations ensured that this type of inactivation induced only subtle spectral changes with negligible influence on the quality of the diagnosis. Furthermore, pilot tests on nonpathogenic strains were systematically conducted to ensure the suitability of sample preparation and to optimize and standardize the workflow for microbial identification. The analysis of the microbial mass spectra was carried out by the individual laboratories on the basis of spectral libraries available on site. All mass spectra were also tested against an in-house HPB library at the Robert Koch Institute (RKI). The averaged identification accuracy was 77% in the first case and improved to >93% when the spectral diagnoses were obtained on the basis of the RKI library. The compilation of complete and comprehensive databases with spectra from a broad strain collection is therefore considered of paramount importance for accurate microbial identification.

Highly pathogenic bacteria (HPB) are risk group 3 bacteria, which are defined as biological agents that can cause severe human disease and present a serious hazard to health care workers. This may present a risk of spreading to the community, but there is usually effective prophylaxis or treatment available (1). To this group belong bacteria such as *Bacillus anthracis*, *Francisella tularensis* subsp. *tularensis* (type A), *Yersinia pestis*, species of the *Brucella melitensis* group, *Burkholderia mallei*, and *Burkholderia pseudomallei*. HPB have the potential to be used in bioterrorist attacks (2, 3). The Centers for Disease Control and Prevention (CDC, Atlanta, GA) have classified *B. anthracis*, *F. tularensis*, and *Y. pestis* as category A and *Brucella* species, *B. mallei*, *B. pseudomallei*, and *Coxiella burnetii* as category B, comprising the main pathogens of concern for use in bioterrorist attacks (4). These pathogens may cause anthrax, tularemia, plague, brucellosis, glanders, melioidosis, and Q fever, respectively. In most parts of the world, the natural prevalence of these agents is low, even though some of these agents cause outbreaks in human and animal populations from time to time (5–8). The intentional release of these agents, however, can result in severe public health consequences, as was shown in the United States in 2001 (9, 10). Therefore, accurate assays for microbial identification are important to ensure proper medical intervention, either in the case of a natural outbreak or an intentional release. Such assays must be able to identify unambiguously a broad panel of potential threat microorgan-

isms in different background matrices that may or may not be contaminated with non-HPB bacteria (11).

Matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry (MS) is a rapid, accurate, sensitive, and cost-effective method that offers an adequate alternative to genome-based approaches and that has been widely used for identification and typing of microorganisms in a clinical routine setup (12–19), as well as for HPB (20–27). This method does

Received 27 March 2015 Returned for modification 21 April 2015

Accepted 1 June 2015

Accepted manuscript posted online 10 June 2015

Citation Lasch P, Wahab T, Weil S, Pályi B, Tomaso H, Zange S, Kiland Granerud B, Drevinek M, Kokotovic B, Wittwer M, Pflüger V, Di Caro A, Stämmler M, Grunow R, Jacob D. 2015. Identification of highly pathogenic microorganisms by matrix-assisted laser desorption ionization–time of flight mass spectrometry: results of an interlaboratory ring trial. J Clin Microbiol 53:2632–2640. doi:10.1128/JCM.00813-15.

Editor: R. Patel

Address correspondence to Daniela Jacob, JacobD@rki.de.

Supplemental material for this article may be found at <http://dx.doi.org/10.1128/JCM.00813-15>.

Copyright © 2015, American Society for Microbiology. All Rights Reserved.

doi:10.1128/JCM.00813-15

not depend on exclusive consumables and has revealed high levels of reproducibility in both intralaboratory and interlaboratory studies (28, 29). Whole cells, crude cell lysates, or bacterial extracts can be utilized to generate taxon-specific fingerprint signatures (30). For safety reasons, the application of MALDI-TOF MS for HPB requires complete inactivation of the microbial samples unless the mass spectrometer is operated in a biosafety level 3 (BSL-3) laboratory. As this is often impossible, whole-cell preparations or crude cell lysates cannot be used for MS-based analyses of HPB.

In this paper, we describe an international exercise for identification of HPB by MALDI-TOF MS which was carried out in the framework of the European Union (EU)-funded project Quality Assurance Exercises and Networking on the Detection of Highly Infectious Pathogens (QUANDHIP). The aim of this Joint Action (JA) was to build up a stabilized consortium that links up 37 highly specialized laboratories from 22 European countries and to guarantee universal exchange of the best diagnostic strategies to support a joint European response to outbreaks of highly pathogenic infectious agents. The JA provided a supportive European infrastructure and strategy for external quality assurance exercises (EQAE), training, and biosafety/biosecurity quality management. The aim of this EQAE was (i) to evaluate the current state of the MALDI-TOF MS-based identification technique for highly pathogenic agents in Europe, (ii) to explore opportunities to advance the diagnostic capabilities, including optimization and standardization of the diagnostic workflow and exchange of standards and protocols (e.g., for verification of MS-compatible inactivation methods), and (iii) to implement measures to improve MALDI-TOF MS-based diagnostics of HPB in Europe (capacity building). The exercise was conducted as a blinded interlaboratory study with 10 different bacterial isolates representing five HPB and five non-HPB test strains and involved in the preparatory-phase pilot tests on non-HPB and inactivation tests with γ -irradiated microorganisms. Eleven QUANDHIP project partners from nine European countries participated in this exercise, including three laboratories from Germany and one each from Austria, the Czech Republic, Denmark, Hungary, Italy, Norway, Sweden, and Switzerland.

MATERIALS AND METHODS

Microbial strains and isolates. All microbial strains originated from the international QUANDHIP strain collection deposited at the Highly Pathogenic Microorganisms Unit (ZBS 2) at the Robert Koch Institute (RKI) in Berlin. These strains represent mainly patient isolates sent by the participating laboratories to the QUANDHIP strain collection. All strains were characterized twice, first in the laboratories that provided the strains and second at RKI/ZBS 2 by means of a large variety of different methods, including classical microbiological, PCR-based, and genotyping methods. An overview of the strains and isolates used in this study is given in Table 1. All microbial strains and isolates were handled according to the respective biosafety regulations outlined in *Technical Rule for Biological Agents 100* (TRBA 100: *Protective Measures for Activities Involving Biological Agents in Laboratories*) (31). HPB and *F. tularensis* subsp. *holarctica* (type B; risk group 2), a very close relative of *F. tularensis* subsp. *tularensis* (type A; risk group 3), were handled according to the requirements of TRBA-100 in a BSL-3 laboratory. The strains were grown under optimal aerobic or microaerophilic conditions on Columbia blood agar plates from Oxoid, Wesel, Germany (*Bacillus* spp., *Yersinia* spp., *Burkholderia* spp., *Brucella* spp., and *Ochrobactrum* spp.) or on heart cysteine agar (HCA) plates (*Francisella* spp.) for at least 24 h and up to 72 h at 37°C. HCA agar plates were produced in a house from an agar base obtained from Bestbion dx

TABLE 1 Overview of microbial strains and species used in the interlaboratory ring trial

Sample	Strain ^a	Concn (CFU/ml)
1	<i>Burkholderia pseudomallei</i> A101-10	1.1×10^9
2	<i>Francisella tularensis</i> subsp. <i>holarctica</i> Ft 32	1.7×10^{10}
3	<i>Brucella canis</i> A183-5	1.9×10^{10}
4	<i>Bacillus anthracis</i> AMES	6.4×10^7
5	<i>Ochrobactrum anthropi</i> A148-11	2.0×10^{10}
6	<i>Yersinia pseudotuberculosis</i> type III	1.3×10^9
7	<i>Burkholderia mallei</i> A106-3	1.0×10^9
8	<i>Burkholderia thailandensis</i> E125	5.6×10^{10}
9	<i>Yersinia pestis</i> A106-2	1.3×10^7
10	<i>Bacillus thuringiensis</i> DSM350	8.6×10^8

^a *Escherichia coli* RKI A139 and *Bacillus cereus* BW-B were utilized for γ -inactivation test measurements in advance of the ring trial. *Bacillus cereus* ATCC 10987, *Bacillus thuringiensis* DSM 5815, *Burkholderia thailandensis* DSM 13276, and *Yersinia enterocolitica* DSM 4780 were used for pilot tests on non-HPB.

(Cologne, Germany) and sheep blood (Oxoid). Except for *Francisella* species isolates, all strains were transferred once onto tryptic soy agar (TSA; VWR, Darmstadt, Germany) or Caso agar (Merck KGaA, Darmstadt, Germany). Cells were harvested from the second passage by resuspending colonies in double-distilled water to an optical density at 600 nm of between 1.0 and 1.2.

Sample preparation/sample inactivation. The concentration of CFU in the microbial suspensions was adjusted to between 10^7 and 10^{10} CFU per ml (Table 1). The suspensions were stored at -75°C until further treatment. Inactivation of microbial samples was carried out by means of high-dose gamma irradiation. For this purpose, microbial suspensions were sent on dry ice from the RKI to Synergy Health Radeberg GmbH (Radeberg/Germany) in accordance with the Dangerous Goods Regulations for category A organisms with UN 2814 (60). Irradiation was carried out according to the recommendations of ISO 11137 (61) using a ^{60}Co gamma ray source. The measured irradiation dose varied between 27.34 and 32.68 kGy. To minimize the possible radiation-associated spectral changes (thermal degradation), the samples were transported and irradiated in the frozen state. For this purpose, all samples were shipped along with a large amount of dry ice. After sample return, it could be verified that a sufficient amount of dry ice was still present and that the samples were not thawed at any time. Tests for sterility after irradiation were conducted by cultivation. In these tests, 10% (vol) of each overall sample solution was added to in-house-produced tryptic soy broth (TSB; Oxoid, Wesel, Germany). Additionally, 100 μl of each sample was twice plated onto the appropriate medium, usually Columbia blood agar or HCA plates (*Francisella*). Incubation for growth in TSB was carried out over a time span of 14 days. Final culturing was performed on Columbia blood agar or HCA plates (*Francisella*) if visible turbidity of TSB was not observable. All agar plates were incubated under species-specific ideal conditions for 3 to 7 days. For the EQAE, only samples which showed no growth after gamma irradiation, either in TSB or on Columbia blood agar or HCA plates, were used.

The inactivated microbial samples were aliquoted (1 ml) and stored again at -75°C until shipment. The aliquots were shipped to the 11 partner institutions on dry ice. Before shipment, blinded MALDI-TOF MS test measurements were performed at the Proteomics and Spectroscopy Unit (ZBS 6) to assess the suitability for MALDI-TOF MS.

When setting up their own spectral databases prior to the ring trial, all partners could choose among a large variety of procedures, protocols, and parameters of sample preparation and data acquisition. While some participants routinely utilize the so-called direct transfer method (30, 32) and/or the ethanol-formic acid (FA) protocol recommended by Bruker Daltonics (30, 33), the group at RKI primarily uses the trifluoroacetic acid (TFA) inactivation/sample preparation method (34). A large advantage of inactivation by gamma irradiation is that this method is compatible with

all of these sample preparation protocols: microbial isolates inactivated by gamma irradiation can in principle be further processed by utilizing any of the different laboratory-specific methodologies. This allowed optimal usage of in-house spectral databases compiled by the individual partner institutions prior to the ring trial. The specific details and settings of the various experimental protocols were polled as a substantial part of the preparation of the ring trial and are summarized in Table S1 in the supplemental material. Furthermore, the preparation of the exercise included systematic MS pilot tests of non-HPB strains by each participating institution. These tests were performed with the aims (i) of identifying and eliminating possible sources of underperformance, such as inadequate procedures of sample preparation or poor parameter selection, and (ii) standardizing, wherever possible, experimental procedures and data acquisition protocols. Within the scope of these pilot tests, MALDI-TOF mass spectra of *Bacillus thuringiensis*, *Burkholderia thailandensis*, *Escherichia coli*, and *Yersinia enterocolitica* were acquired, shared, and jointly analyzed.

MALDI-TOF MS. Details of MALDI-TOF MS measurements are in Table S1 in the supplemental material.

Identification approach A. The analysis of mass spectra from blinded microbial samples was carried out first on-site by the ring trial participants themselves. In this approach, the participants employed different types of identification software and utilized a variety of distinct mass spectral libraries, such as Bruker's commercial database for clinical microbiology, the standard BioTyper database, the so-called Security Relevant reference library (SR library) from Bruker, the SARAMIS database, and in-house databases compiled by the institutions themselves (see Table S1 in the supplemental material). During EQAE's preparatory stage, some of the ring trial participants initiated data exchange activities with the purpose of increasing the size and improving the degree of coverage of these in-house libraries.

Identification approach B. After submission of the identification results, all mass spectra were collected at the study center (RKI) and subsequently analyzed for a second time using the database of HPB at RKI. This in-house database consists of 1,118 entries (main spectral projections [MSPs]), each corresponding to a defined microbial strain from the genera *Bacillus*, *Burkholderia*, *Brucella*, *Francisella*, *Vibrio*, and *Yersinia* (along with a number of clinically relevant species from the genera *Escherichia*, *Enterococcus*, *Staphylococcus*, *Streptococcus*, and others). These MSPs represent database entries in the server component of Bruker's BioTyper software package which can be queried via BioTyper software clients (version 3.1, build 66; Bruker). Microbial identification was achieved on the basis of the unmodified standard BioTyper identification method compiled by the manufacturer. Furthermore, identification was conducted by means of logarithmic scores with cutoff values as specified by Bruker: log score values larger than 2.3 are required for a reliable (highly probable) identification on the species level, and scores of between 2.3 and 2.0 represent probable species identification. Scores of between 2.0 and 1.7 point toward a reliable genus identification, while values below 1.7 are regarded as unreliable (35). Due to the proprietary nature of the spectral data file format, analysis in identification approach B was limited to spectra acquired by mass spectrometers produced by Bruker: the BioTyper client software does not allow importing data in a format other than the Bruker format. As one of the participating institutions employs MS equipment produced by Shimadzu (laboratory XI), identification approach B involved the analyses of MS data from 10 of the 11 participating institutions.

Identification approach C. In the third analysis approach, the Matlab-based software solution MicrobeMS version 0.72 (24, 36–39) developed at RKI was used (Matlab; The Mathworks, Inc., Natick, MA). MicrobeMS is publicly available as Matlab p-code (free of charge) and provides direct access to Bruker's raw spectral data and to spectra acquired by the Vitek MS workflow (formerly SARAMIS, bioMérieux/Shimadzu) via the mzXML data format (40). The software allows spectral preprocessing, such as smoothing, baseline correction, intensity normalization, and internal calibration, and can be employed to produce reference peak

lists from microbial MALDI-TOF mass spectra (39). Furthermore, MicrobeMS can be used to systematically screen for taxon-specific biomarkers and for visualization of large spectral data sets (via pseudogel views). Within the context of the present study, the software was utilized for identification purposes in combination with the mass spectral database for HPB. This allowed cross-platform analysis of microbial mass spectra from partner institutions using instrumentation from two different manufacturers, Bruker and bioMérieux/Shimadzu (see reference 39 for details).

RESULTS AND DISCUSSION

Gamma inactivation. Complete inactivation of all pathogens prior to dispatch to the ring trial participants was considered an essential prerequisite for successful implementation of the inter-laboratory ring trial. Although it would in principle have been possible to distribute viable BSL-3 pathogens throughout Europe, the very strict legal provisions would have represented a significant organizational challenge with very high shipment costs. The shipment of viable BSL-3 samples is only allowed as infectious material (class 6.2) category A in accordance with the Dangerous Goods Regulations, whereas inactivated material can be dispatched very easily.

As stated earlier, gamma irradiation was selected as the inactivation method of choice. Although the TFA sample preparation protocol has been specifically developed as a MALDI-TOF MS-compatible method for microbial inactivation of HPB, it was decided not to employ this protocol. It is well-known that spectra produced by acid-based methods exhibit systematic changes compared to spectra created by the direct transfer method (41). Differences between spectra obtained by the ethanol-FA and the TFA method, however, are much smaller, since both techniques are ultimately based on acid extraction. In any case, shipment of γ -inactivated biological material allowed the partners to choose the appropriate preparation protocol, which resulted in a very high degree of compatibility with existing in-house database solutions at the partner institutions.

High-dose gamma irradiation is known in the literature as a method suitable for reliably inactivating bacterial pathogens (42, 43) while leaving the primary protein structures basically intact. Our comparative measurements of pathogenic and nonpathogenic microbial strains essentially confirmed the data in the literature: identification was successful after high-dose gamma irradiation, but irradiation resulted in slightly lower BioTyper log score values (data not shown). Under the specific experimental conditions at RKI, it was found that the signals relevant for identification remained very marked after gamma irradiation, though with reduced peak intensities. The MALDI-TOF mass spectra of *E. coli* and *B. cereus* exemplarily demonstrate the presence of all of the main peaks in both the irradiated and the reference samples (Fig. 1). However, the spectra of the γ -inactivated samples generally exhibited a lower signal-to-noise ratio due to the slightly reduced peak intensities.

Pilot tests on non-HPB strains. Pilot tests were conducted by the partners to identify factors that affect the overall performance of the MS-based identification technique and to standardize experimental procedures, data acquisition protocols, and methods of spectral analysis. In the context of the preparation of the pilot tests, experimental methods and parameters were polled (see Table S1 in the supplemental material).

The jointly conducted analysis of microbial MALDI-TOF mass spectra from non-HPB revealed a number of peculiarities, such as

FIG 1 MALDI-TOF mass spectra of control samples (black traces) and microorganisms inactivated by means of high-dose gamma irradiation (red traces). Irradiated samples of *E. coli* A139 and *B. cereus* BW-B (spores) were prepared for MALDI-TOF MS in the same way as the retained control samples, by means of the TFA inactivation method (34). The spectra (smoothed and baseline corrected) demonstrate only insignificant differences between the irradiated and control samples, suggesting that gamma irradiation is compatible with the routine sample preparation protocols used by the partner institutions (see the text for details). Intens. [a.u.], intensity in arbitrary units.

broadened peaks, spectrum baseline irregularities (elevated baselines), and the appearance of additional satellite peaks in some of the microbial mass spectra. While peak broadening and baseline elevation effects could be identified relatively easily as a result of the application of excessive laser power (see reference 44), it was more challenging to identify the sources and causes of additional satellite peaks.

Satellite peaks. In Fig. 2, the bottom panel illustrates a first example of satellite peaks in a mass spectrum of *B. thuringiensis*. As shown, additional peaks such as are described above occurred at 16-Da intervals at higher molecular masses with respect to the parent peak (cf. peak series at m/z 4,335, 4,351, and 4,367). The spectrum of *B. thuringiensis* obtained by the reference sample preparation method (TFA inactivation) clearly demonstrates

FIG 2 Oxidation of *Bacillus thuringiensis* microbial extracts by sodium hypochlorite (NaClO). Top, reference mass spectrum of a *B. thuringiensis* sample prepared on the basis of the trifluoroacetic acid (TFA) inactivation technique (34). Bottom, TFA-treated sample of the same *Bacillus* strain with likely contamination by sodium hypochlorite. The spectral differences—satellite peaks at +16-Da intervals—are attributed to contamination by the oxidant NaClO, which was employed for external sterilization of sample vials during outward transfer from a BSL-3 laboratory (spectra were smoothed and baseline corrected; see the text for further details).

the absence of such peaks in the control measurements (Fig. 2, top). The observed satellite peaks are most likely caused by the action of sodium hypochlorite (NaClO), which is known as an effective disinfectant and a strong oxidizing agent. Because of its well-known antimicrobial and sporicidal properties, NaClO was applied in the laboratory of one of the partners for 15 min in a concentration of 10% (vol/vol) for external sterilization of the MALDI-TOF MS sample vials. It seems likely that during this period, small amounts of NaClO entered the tubes, e.g., via incompletely closed lids. In proteins, the amino acid methionine and aromatic residues like tryptophan and tyrosine are potential first oxidation targets (45, 46). In the case of oxidation of methionine, the experimentally observed mass differences between the parent and satellite peaks of 16 Da would fit well with the computed masses of unoxidized methionine and methionine sulfoxide as the singly oxidized species (47). However, the mass differences mentioned would be also observable in the case of oxidation of other amino acids.

Similar oxidation-induced satellite peaks ($\Delta m/z$ of +16 Da) were observed when microbial samples were accidentally inoculated with another sterilizing agent, peracetic acid (PAA). PAA also acts as an oxidizing agent and can cause the oxidation of lipids and amino acid side chains of peptides and small proteins in microbial extracts (data not shown).

Satellite peaks were also detected in samples prepared by means of the ethanol-FA sample preparation protocol (30, 33). Using the examples of spectra from *B. cereus* ATCC 10987 and *B. thuringiensis* DSM 5815, the traces in Fig. 3 show the presence of additional peaks at a distance of 28 Da: the black curves denote mass spectra in the m/z 6,250-to-7,500 region of *Bacillus* samples prepared by the TFA inactivation method, while the red spectra were obtained from identical *Bacillus* strains prepared by means of the ethanol-FA sample preparation method, which included incubation in 70% FA (vol/vol) for 30 min. Both pairs of spectra display parent peaks at m/z 6,695 (*B. cereus*) or m/z 6,711 (*B. thuringiensis*), assigned as a beta-type small, acid-soluble protein (β -SASP), m/z 6,835 (α -SASP), and m/z 7,082 (α - β -SASP) (see references 24 and 48 for peak assignments). Apart from these dominating signals, the spectra of FA-treated samples exhibit additional satellite peaks at m/z 6,723 (*B. cereus*) or m/z 6,739 (*B. thuringiensis*) and at m/z 6,863 (both strains). Satellite signals are found at a distance of +28 Da from the parent peaks, typically with intensities of less than 20% of the original signal. A likely explanation for the occurrence of satellite peaks would be chemical modification of the SASPs (formyl esterification) due to prolonged sample treatment by FA. FA treatment has been associated with formylation of proteins in microbial extracts (49), with the specific targets of serine and threonine residues. Furthermore, it is known that formylation is particularly effective when highly concentrated FA is applied to small hydrophobic proteins (50), such as SASPs. Since each additional satellite peak may potentially have a negative impact on the performance of the identification algorithm, the exposure time to FA should be minimized whenever possible. Taking into account that this note is also given in the BioTyper manual (see reference 35), the reduction of FA incubation time is considered an important measure for improving the accuracy of identification.

Results of the interlaboratory ring trial. Table S2 in the supplemental material has a summary of the identification results in the context of identification approach A. This approach involved

FIG 3 Formylation of spore marker proteins (small, acid-soluble proteins [SASPs]) in test samples of *Bacillus cereus* and *Bacillus thuringiensis* possibly as a result of prolonged treatment by highly concentrated (70%) formic acid (FA) (24). Hash marks (#) indicate peaks at m/z 6,695 or 6,711 corresponding to two possible variants of β -SASP in *B. cereus* and *B. thuringiensis*. Ampersands (&) indicate peaks at m/z 6,835 (α -SASP in *B. cereus* ATCC 10987; UniProt Q73CW6). All mass spectra were smoothed, baseline corrected, and intensity normalized. Black traces are reference MALDI-TOF mass spectra of *Bacillus* samples prepared by the TFA inactivation method (34). Red traces are spectra from identical strains processed by the ethanol-FA method (33). Red numbers denote additional mass peaks at a distance of +28 Da with reference to the α -SASP (m/z 6,835) or the β -SASP (m/z 6,695/6,711) peaks.

data analysis on site by each partner institution. The table not only shows an overview of the results of the blinded identity tests, it also provides either the logarithmic BioTyper scores or, alternatively, the respective SARAMIS score values. In approach A, MALDI-TOF mass spectra acquired by laboratory XI were analyzed twice, first by using customized in-house algorithms and second by an analysis carried out elsewhere by means of the SARAMIS software and the database solution from Anagnostec. For this reason, Table S2 includes an additional column headed "Laboratory XII," which is different from identification approaches B and C.

The color scheme used in Table S2 is a traffic light coloring scheme: it uses the colors green for correct, yellow for partially correct, and red for false identification results. A correct result was obtained when the identity was accurately revealed at the genus, species, and subspecies levels. Cells colored yellow denote identification results which were either incomplete—for example, in cases where the subspecies specification was lacking (see sample 2, *F. tularensis* subsp. *holarctica*, in Table S2)—or where the genus assignment was correct but the species was left unassigned (e.g., in lines 9 and 10, laboratory VIII, *Yersinia* sp. and *Bacillus* sp., in Table S2). Furthermore, a result was also considered partially correct in cases of contradictory identification results, i.e., if different

No. Sample Identity	Identification Approach A			Identification Approach B			Identification Approach C		
	Correct	Partly correct	Incorrect	Correct	Partly correct	Incorrect	Correct	Partly correct	Incorrect
1 <i>Burkholderia pseudomallei</i> A101-10	9	1	1	9	1	0	10	1	0
	9.5 (86%)			9.5 (95%)			10.5 (95%)		
2 <i>Francisella tularensis</i> subsp. <i>holarctica</i> Ft 32	4.5	6.5	0	9	1	0	11	0	0
	7.75 (70%)			9.5 (95%)			11 (100%)		
3 <i>Brucella canis</i> A183-5	3	8	0	10	0	0	11	0	0
	7 (64%)			10 (100%)			11 (100%)		
4 <i>Bacillus anthracis</i> AMES	9	0	2	9	1	0	11	0	0
	9 (82%)			9.5 (95%)			11 (100%)		
5 <i>Ochrobactrum anthropi</i> A148-11	10	1	0	10	0	0	11	0	0
	10.5 (95%)			10 (100%)			11 (100%)		
6 <i>Yersinia pseudotuberculosis</i> type III	8	0	3	6	3	1	9	1	1
	8 (73%)			7.5 (75%)			9.5 (86%)		
7 <i>Burkholderia mallei</i> A106-3	9	0	2	9	1	0	8	3	0
	9 (82%)			9.5 (95%)			9.5 (86%)		
8 <i>Burkholderia thailandensis</i> E125	10	1	0	10	0	0	11	0	0
	10.5 (95%)			10 (100%)			11 (100%)		
9 <i>Yersinia pestis</i> A106-2	7	3	1	6	4	0	6	5	0
	8.5 (77%)			8 (80%)			8.5 (77%)		
10 <i>Bacillus thuringiensis</i> DSM350	4	2	5	10	0	0	9	2	0
	5 (45%)			10 (100%)			10 (91%)		

FIG 4 Summary of the different identification results from the MALDI-TOF MS ring trial, with the numbers of correct, partly correct, and incorrect identifications, as well as point scores for identification (correct identification, one point; partly correct, half a point; incorrect, zero points) and the corresponding identification accuracy values (percentages). Green, identification accuracy for the given microbial strain is equal to or greater than 90%; yellow, accuracy is equal to or greater than 75% and below 90%; red, accuracy is below 75%.

microbial identities were obtained from spectra of technical replicate measurements. In such cases, however, at least one result had to be correct. An example of contradictory identification results can be found in Table S3 for sample 6 from laboratory X. Score values in this or similar instances were indicated by a range of values. Identification results were considered incorrect (red cells) if either an HPB was clearly assigned as a non-HPB (false negative) or, alternatively, if a non-HPB was identified as an HPB (false positive). Cases where no false-positive/false-negative results were obtained, for example, if a result was inconsistent or unavailable (no spectrum), were also regarded as partially correct (no confirmation, but also no all clear). To calculate the overall accuracy index of the entire identification approach, a point system was introduced, giving one point for each correct identification result (green). Furthermore, cells with partially correct results (yellow) received half points, while no points were given for incorrect results (red). All points were then summed over the entire table; the sums were subsequently divided by the number of cells of each table. The quotient thus determined was finally multiplied by 100 and expressed as a percentage. To exclude an undue weighting of the measured data from laboratory XI, the point values from the rows "Laboratory XI" and "Laboratory XII" were averaged before summation in identification approach A.

The overall identification accuracy of identification approach A was 77% (see Table S2 in the supplemental material). While the accuracy of identification of samples 1 (*B. pseudomallei*), 4 (*B. anthracis*), 5 (*Ochrobactrum anthropi*), 7 (*B. mallei*), 8 (*B. thailandensis*), and 9 (*Y. pestis*) was relatively high, there were major problems when diagnosing samples 2 (*F. tularensis* subsp. *holarctica*), 3 (*Brucella canis*), 6 (*Yersinia pseudotuberculosis*), and 10 (*B. thuringiensis*). Furthermore, the results from laboratory IX were generally difficult to assess. In this laboratory, diagnoses were made only on the basis of the standard BioTyper database for

clinical microorganisms; neither an in-house database of HPB nor the SR library from Bruker were available to this partner (cf. Tables S1 and S2).

The overall identification results improved significantly when spectra of the interlaboratory exercise were tested against the database of highly pathogenic microorganisms compiled at RKI over the past 10 years; the overall identification accuracy improved from 77.0% in approach A to 93.5% in approach B (see Table S3 in the supplemental material). The improvements were particularly striking in the cases of samples 2 (*F. tularensis* subsp. *holarctica*), 3 (*B. canis*), and 10 (*B. thuringiensis*). However, differentiation between samples 6 (*Y. pseudotuberculosis*) and 9 (*Y. pestis*) improved only slightly in approach B.

In the third approach, identification approach C, the overall picture did not differ much from approach B (see Table S4 in the supplemental material). The minor improvement in the overall identification accuracy (93.7%, compared to 93.5%) is statistically insignificant, which is not particularly surprising: although both approaches involved different software implementations with different algorithms, they relied on an identical spectral database. The results given in Table S4 demonstrate a decreased identification rate for sample 7 (*B. mallei*) and a slight improvement for sample 6 (*Y. pseudotuberculosis*). However, the major advantage of approach C over approach B consists in the fact that it allows analysis of spectra obtained by means of the bioMérieux/Shimadzu system (cf. rows "Laboratory XI" of Tables S3 and S4). Due to missing import capabilities for Shimadzu-specific spectral files in the BioTyper software, the data acquired by laboratory XI may be analyzed by approach C but not using the BioTyper software employed in identification approach B.

Figure 4 shows a summary of the results of all identification approaches. This table again illustrates the improvements of the

identification accuracies in approaches B and C in comparison to those of approach A, particularly for samples 1 to 4 and 10. With regard to samples 2 (*F. tularensis* subsp. *holarctica*) and 3 (*B. canis*), we assume that the relatively high error rates in approach A derive from incomplete or missing spectral entries for both subspecies/species in the SR BioTyper library extension. We have noted that identification of *F. tularensis* subsp. *holarctica* and of *B. canis* was incomplete in cases where identification was made by means of this particular database extension. A closer examination of the SR database content revealed the absence of subspecies information in entries for *F. tularensis* (sample 2) and the lack of spectral entries for *Brucella* species other than *B. melitensis* (sample 3).

In contrast, it was interesting to note that the sophisticated software algorithms employed in approaches B and C can cause problems even in cases where extensive spectral databases are available. To give an example, differentiation between *Y. pseudotuberculosis* and *Y. pestis* by approaches B and C is far from being ideal (cf. samples 6 and 9 in Tables S3 and S4 in the supplemental material). To a certain extent, this could be caused by the low initial concentration of *Y. pestis* in the sample solution (1.3×10^7 CFU/ml) (Table 1). Several ring trial participants have indeed reported a relatively poor signal-to-noise ratio in MALDI-TOF mass spectra acquired from aliquots of sample 9. Low spectral quality is certainly a factor which makes differentiation of *Y. pestis* and *Y. pseudotuberculosis* difficult. An even more important factor, however, is the very high degree of similarity of spectra from these two very closely related species. In fact, *Y. pestis* is known as a clone of *Y. pseudotuberculosis* which has only recently evolved from *Y. pseudotuberculosis* (51, 52). Both species share genomic sequences and have identical 16S rRNA genes (53). As a consequence, their differentiation by MALDI-TOF MS is challenging; it has been found that differentiation can be carried out only on the basis of a single mass peak at m/z 3,065 (36, 38). This peak has been assigned to a fragment of the plasmid-encoded (pPCP1) Pla protein. Therefore, MS-based differentiation is possible only for strains of *Y. pestis* carrying the pPCP1 plasmid. At this point, it should be stressed that visual inspection of the mass spectra would have helped in solving the particular problem of differentiating *Y. pseudotuberculosis* and *Y. pestis*. Although the biomarker for *Y. pestis* at m/z 3,065 is typically very intense, pattern recognition routines do not always provide reliable results in cases when the outcome of the identification is based on the presence or absence of only a single biomarker. Following this line of reasoning, the supervised modeling approach chosen by laboratory XI, which relies on 15 biomarkers to discriminate between *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*, may provide the basis for a more robust typing scheme (54).

In the present study, problems also occurred when differentiating the closely related members of the *B. cereus* group, *B. anthracis*, *B. cereus*, and *B. thuringiensis*. First, we have no information on whether MALDI-TOF MS allows reliable differentiation of *B. cereus* and *B. thuringiensis*. Our own observations, however, revealed that strains from both species are frequently identified based on their strain-specific spectral profiles. On the other hand, mass spectra of *B. anthracis* strains exhibit a specific β -SASP signal at m/z 6,679 (22, 24, 55–58) that is usually not present in spectra of other *B. cereus* group members. However, in the recent literature, there is increasing evidence that spectra of certain strains of *B. cereus* and *B. thuringiensis* may also exhibit β -SASP peaks at m/z

6,679 (59) (see also the spectrum of *B. cereus* BW-B in Fig. 1). Therefore, this β -SASP biomarker is not necessarily pathognomonic for *B. anthracis*. Furthermore, we and others have noted that the second published biomarker of *B. anthracis*, at m/z 5,413 (24), is often found in spectra of *B. cereus* and *B. thuringiensis* also. Both facts should be considered when assessing the identification results for *B. cereus* group members: the results of MALDI-TOF MS should not form the sole basis for potentially far-reaching decisions; for example, in the event of suspected intentional release of *B. anthracis*.

Conclusions. This paper reports on an interlaboratory external quality assurance exercise (EQAE) conducted by 11 partner institutions from nine European countries. In this ring trial, MALDI-TOF MS was used as a tool for rapid, reliable, and cost-effective identification of highly pathogenic microorganisms. In the preparatory phase of the exercise, pilot tests on nonpathogenic strains were carried out in order to optimize and standardize the experimental procedures at the partner institutions and to identify possible sources of underperformance. Irradiation by gamma rays proved to be a MALDI-TOF MS-compatible inactivation method which induced only subtle spectral changes with negligible influence on the quality of the diagnosis. In the ring trial, the average identification accuracy was 77% when using nonstandard mass spectral databases. The accuracy improved to >93% when spectral diagnoses were reached on the basis of an optimized spectral database with better coverage of highly pathogenic and related species.

The present EQAE has highlighted current strengths and weaknesses of the MALDI-TOF MS-based approach for identification of HPB and has confirmed the need for high-quality spectral databases to facilitate improved identification accuracy. Experiences gathered from the present international EQAE also suggest that, as long as high-quality and comprehensive spectral databases are available, different preparative procedures, the degree of user experience, and different types of instrumentation and analysis software are not likely to critically affect identification of HPB. The compilation of complete and comprehensive databases is thus considered to be of paramount importance for reaching accurate and reliable spectral diagnoses. Future efforts to improve the diagnostic capabilities should therefore focus on the exchange of validated reference spectra. We are confident that further ring trials will confirm the improvements achieved by such activities.

ACKNOWLEDGMENTS

We thank T. M. Fuchs (ZIEL, Technical University Munich, Germany), J. Rau (CVUA, Stuttgart, Germany), W. Beyer (University of Hohenheim, Stuttgart, Germany), A. Paauw (TNO, Rijswijk, Netherlands), M. Dybwad (NDRE, Kjeller, Norway), and N. Schürch (Labor Spiez, BABS, Spiez, Switzerland) for providing strains, samples, or spectra of important microbial pathogens. S. Weil, S. Zange, and B. Pályi are grateful to P. Hufnagl (AGES, Vienna, Austria), B. Thoma (InstMikroBioBw, Munich, Germany), and M. Iván (Sемmelweis University, Budapest, Hungary), respectively. In addition, we thank S. Becker, P. Lochau, A. Schneider, S. Howaldt, and R. Andrich (all RKI, Berlin, Germany) for excellent technical assistance.

We are very grateful to the European Commission and CHAFAE for financially and technically supporting the QUANDHIP Joint Action (CHAFAE grant agreement number 2010 21 02). Parts of this work were supported by the Federal Ministry of Education and Research, BMBF (Förderkennzeichen/grant number 13N11166).

REFERENCES

- Anonymous. 2000. Reference directive 2000/54/EC of the European Parliament and of the Council of 18 September 2000 on the protection of workers from risks related to exposure to biological agents at work. Off J Eur Communities L262:21–45.
- Branda JA, Ruoff K. 2002. Bioterrorism. Clinical recognition and primary management. Am J Clin Pathol 117(Suppl):S116–S123.
- Pappas G, Panagopoulou P, Akritidis N. 2009. Reclassifying bioterrorism risk: are we preparing for the proper pathogens? J Infect Public Health 2:55–61. <http://dx.doi.org/10.1016/j.jiph.2009.03.002>.
- Horn JK. 2003. Bacterial agents used for bioterrorism. Surg Infect (Larchmt) 4:281–287. <http://dx.doi.org/10.1089/109629603322419625>.
- Svensson K, Back E, Eliasson H, Berglund L, Granberg M, Karlsson L, Larsson P, Forsman M, Johansson A. 2009. Landscape epidemiology of tularemia outbreaks in Sweden. Emerg Infect Dis 15:1937–1947. <http://dx.doi.org/10.3201/eid1512.090487>.
- Theläus J, Andersson A, Broman T, Backman S, Granberg M, Karlsson L, Kuoppa K, Larsson E, Lundmark E, Lundström JO, Mathisen P, Naslund J, Schafer M, Wahab T, Forsman M. 2014. Francisella tularensis subspecies holarctica occurs in Swedish mosquitoes, persists through the developmental stages of laboratory-infected mosquitoes and is transmissible during blood feeding. Microb Ecol 67:96–107. <http://dx.doi.org/10.1007/s00248-013-0285-1>.
- Vogler AJ, Chan F, Nottingham R, Andersen G, Drees K, Beckström-Sternberg SM, Wagner DM, Chanteau S, Keim P. 2013. A decade of plague in Mahajanga, Madagascar: insights into the global maritime spread of pandemic plague. mBio 4(1):e00623–12. <http://dx.doi.org/10.1128/mBio.00623-12>.
- Vogler AJ, Chan F, Wagner DM, Roumagnac P, Lee J, Nera R, Eppinger M, Ravel J, Rahalison L, Rasoamanana BW, Beckström-Sternberg SM, Achtman M, Chanteau S, Keim P. 2011. Phylogeography and molecular epidemiology of Yersinia pestis in Madagascar. PLoS Negl Trop Dis 5:e1319. <http://dx.doi.org/10.1371/journal.pntd.0001319>.
- Bartlett JG, Inglesby TV, Jr, Borio L. 2002. Management of anthrax. Clin Infect Dis 35:851–858. <http://dx.doi.org/10.1086/341902>.
- Kennedy H. 31 October 2001. Daschle letter bombshell—billions of anthrax spores. New York Daily News, New York, NY. <http://www.nydailynews.com/archives/news/daschle-letter-bombshell-billions-anthrax-spores-article-1.921507>.
- Wagar EA, Mitchell MJ, Carroll KC, Beavis KG, Petti CA, Schlager R, Yasin B. 2010. A review of sentinel laboratory performance: identification and notification of bioterrorism agents. Arch Pathol Lab Med 134:1490–1503. <http://dx.doi.org/10.1043/2010-0098-CP.1>.
- Claydon MA, Davey SN, Edwards-Jones V, Gordon DB. 1996. The rapid identification of intact microorganisms using mass spectrometry. Nat Biotechnol 14:1584–1586. <http://dx.doi.org/10.1038/nbt1196-1584>.
- Holland RD, Wilkes JG, Raffi F, Sutherland JB, Persons CC, Voorhees KJ, Lay JO, Jr. 1996. Rapid identification of intact whole bacteria based on spectral patterns using matrix-assisted laser desorption/ionization with time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 10:1227–1232. [http://dx.doi.org/10.1002/\(SICI\)1097-0231\(19960731\)10:10<1227::AID-RCM659>3.0.CO;2-6](http://dx.doi.org/10.1002/(SICI)1097-0231(19960731)10:10<1227::AID-RCM659>3.0.CO;2-6).
- Krishnamurthy T, Rajamani U, Ross PL. 1996. Detection of pathogenic and non-pathogenic bacteria by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 10:883–888. [http://dx.doi.org/10.1002/\(SICI\)1097-0231\(19960610\)10:8<883::AID-RCM594>3.3.CO;2-M](http://dx.doi.org/10.1002/(SICI)1097-0231(19960610)10:8<883::AID-RCM594>3.3.CO;2-M).
- Dieckmann R, Helmuth R, Erhard M, Malorny B. 2008. Rapid classification and identification of salmonellae at the species and subspecies levels by whole-cell matrix-assisted laser desorption/ionization-time of flight mass spectrometry. Appl Environ Microbiol 74:7767–7778. <http://dx.doi.org/10.1128/AEM.01402-08>.
- Sandrin TR, Goldstein JE, Schumaker S. 2013. MALDI-TOF MS profiling of bacteria at the strain level: a review. Mass Spectrom Rev 32:188–217. <http://dx.doi.org/10.1002/mas.21359>.
- Fenselau C, Demirev PA. 2001. Characterization of intact microorganisms by MALDI mass spectrometry. Mass Spectrom Rev 20:157–171. <http://dx.doi.org/10.1002/mas.10004>.
- Patel R. 2015. MALDI-TOF MS for the diagnosis of infectious diseases. Clin Chem 61:100–111. <http://dx.doi.org/10.1373/clinchem.2014.221770>.
- Patel R. 2013. Matrix-assisted laser desorption/ionization-time of flight mass spectrometry in clinical microbiology. Clin Infect Dis 57:564–572. <http://dx.doi.org/10.1093/cid/cit247>.
- Ferreira L, Vega Castaño S, Sánchez-Juanes F, González-Cabrero S, Menegotto F, Orduña-Domingo A, González-Buitrago JM, Muñoz-Bellido JL. 2010. Identification of Brucella by MALDI-TOF mass spectrometry. Fast and reliable identification from agar plates and blood cultures. PLoS One 5:e14235.
- Seibold E, Maier T, Kostrzewa M, Zeman E, Splettstoesser W. 2010. Identification of Francisella tularensis by whole-cell matrix-assisted laser desorption/ionization-time of flight mass spectrometry: fast, reliable, robust, and cost-effective differentiation on species and subspecies levels. J Clin Microbiol 48:1061–1069. <http://dx.doi.org/10.1128/JCM.01953-09>.
- Elhanany E, Barak R, Fisher M, Kobiler D, Altboum Z. 2001. Detection of specific Bacillus anthracis spore biomarkers by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 15:2110–2116. <http://dx.doi.org/10.1002/rcm.491>.
- Drevinek M, Dresler J, Klimentova J, Pisa L, Hubalek M. 2012. Evaluation of sample preparation methods for MALDI-TOF MS identification of highly dangerous bacteria. Lett Appl Microbiol 55:40–46. <http://dx.doi.org/10.1111/j.1472-765X.2012.03255.x>.
- Lasch P, Beyer W, Nattermann H, Stammer M, Siegbrecht E, Grunow R, Naumann D. 2009. Identification of Bacillus anthracis by using matrix-assisted laser desorption/ionization-time of flight mass spectrometry and artificial neural networks. Appl Environ Microbiol 75:7229–7242. <http://dx.doi.org/10.1128/AEM.00857-09>.
- Hagan NA, Lin JS, Antoine MD, Cornish TJ, Quizon RS, Collins BF, Feldman AB, Demirev PA. 2011. MALDI mass spectrometry for rapid detection and characterization of biological threats, p 211–224. In Fenselau C, Demirev P (ed), Rapid characterization of microorganisms by mass spectrometry. ACS Symposium Series no. 1065. American Chemical Society, Washington, DC.
- Demirev PA, Fenselau C. 2008. Mass spectrometry in biodefense. J Mass Spectrom 43:1441–1457. <http://dx.doi.org/10.1002/jms.1474>.
- Cunningham SA, Patel R. 2013. Importance of using Bruker's security-relevant library for Biotyper identification of Burkholderia pseudomallei, Brucella species, and Francisella tularensis. J Clin Microbiol 51:1639–1640. <http://dx.doi.org/10.1128/JCM.00267-13>.
- Mellmann A, Bimet F, Bizet C, Borovskaya AD, Drake RR, Eigner U, Fahr AM, He Y, Ilina EN, Kostrzewa M, Maier T, Mancinelli L, Moussaoui W, Prévost G, Putignani L, Seachord CL, Tang YW, Harsen D. 2009. High interlaboratory reproducibility of matrix-assisted laser desorption/ionization-time of flight mass spectrometry-based species identification of nonfermenting bacteria. J Clin Microbiol 47:3732–3734. <http://dx.doi.org/10.1128/JCM.00921-09>.
- Wittwer M, Lasch P, Drevinek M, Schmoldt S, Indra A, Jacob D, Grunow R. 2012. First report: application of MALDI-TOF MS within an external quality assurance exercise for the discrimination of highly pathogenic bacteria from contaminant flora. Appl Biosaf 17:59–63.
- Freiwald A, Sauer S. 2009. Phylogenetic classification and identification of bacteria by mass spectrometry. Nat Protoc 4:732–742. <http://dx.doi.org/10.1038/nprot.2009.37>.
- Committee for Biological Agents (ABAS). 2013. Technical rule for biological agents 100 (TRBA 100). Protective measures for activities involving biological agents in laboratories. GMBI no. 51/52 of 17.10.2013, p 1010–1042.
- Schulthess B, Bloemberg GV, Zbinden R, Bottger EC, Hombach M. 2014. Evaluation of the Bruker MALDI Biotyper for identification of Gram-positive rods: development of a diagnostic algorithm for the clinical laboratory. J Clin Microbiol 52:1089–1097. <http://dx.doi.org/10.1128/JCM.02399-13>.
- Maier T, Klepel S, Renner Z, Kostrzewa M. 2006. Fast and reliable MALDI-TOF MS-based microorganism identification. Nat Methods 3:324–334. <http://dx.doi.org/10.1038/nmeth0406-324>.
- Lasch P, Nattermann H, Erhard M, Stammer M, Grunow R, Bannert N, Appel B, Naumann D. 2008. MALDI-TOF mass spectrometry compatible inactivation method for highly pathogenic microbial cells and spores. Anal Chem 80:2026–2034. <http://dx.doi.org/10.1021/ac701822j>.
- Anonymous. 2012. MALDI BioTyper 3.0 User Manual. Bruker Daltonics GmbH, Bremen, Germany.
- Lasch P, Drevinek M, Nattermann H, Grunow R, Stammer M, Dieckmann R, Schwecke T, Naumann D. 2010. Characterization of Yersinia using MALDI-TOF mass spectrometry and chemometrics. Anal Chem 82:8464–8475. <http://dx.doi.org/10.1021/ac101036s>.
- Lasch P, Fleige C, Stammer M, Lauer F, Nubel U, Witte W, Werner G.

2014. Insufficient discriminatory power of MALDI-TOF mass spectrometry for typing of *Enterococcus faecium* and *Staphylococcus aureus* isolates. *J Microbiol Methods* 100:58–69. <http://dx.doi.org/10.1016/j.mimet.2014.02.015>.
38. Lasch P, Naumann D. 2011. MALDI-TOF mass spectrometry for the rapid identification of highly pathogenic microorganisms, p 219–212. In Stulik J, Toman R, Butaye P, Ulrich RG (ed), *Proteomics, glycomics and antigenicity of BSL3 and BSL4 agents*, 1st ed. Wiley-VCH Verlag GmbH & Co KGaA, Weinheim, Germany.
39. Lasch P. 2015. MicrobeMS: a Matlab toolbox for analysis of microbial MALDI-TOF mass spectra. <http://www.microbe-ms.com>.
40. Wikipedia contributors. 2015. Mass spectrometry data format, on Wikipedia, The Free Encyclopedia. https://en.wikipedia.org/wiki/Mass_spectrometry_data_format#mzML.
41. Goldstein JE, Zhang L, Borror CM, Rago JV, Sandrin TR. 2013. Culture conditions and sample preparation methods affect spectrum quality and reproducibility during profiling of *Staphylococcus aureus* with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Lett Appl Microbiol* 57:144–150. <http://dx.doi.org/10.1111/lam.12092>.
42. Tracz DM, McCorrister SJ, Westmacott GR, Corbett CR. 2013. Effect of gamma radiation on the identification of bacterial pathogens by MALDI-TOF MS. *J Microbiol Methods* 92:132–134. <http://dx.doi.org/10.1016/j.mimet.2012.11.013>.
43. Dauphin LA, Newton BR, Rasmussen MV, Meyer RF, Bowen MD. 2008. Gamma irradiation can be used to inactivate *Bacillus anthracis* spores without compromising the sensitivity of diagnostic assays. *Appl Environ Microbiol* 74:4427–4433. <http://dx.doi.org/10.1128/AEM.00557-08>.
44. Hillenkamp FE, Peter-Katalinic PE. 2013. MALDI MS: a practical guide to instrumentation, methods and applications, 2nd ed. Wiley-VCH Verlag GmbH & Co KGaA, Weinheim, Germany.
45. Berlett BS, Levine RL, Stadtman ER. 1996. Comparison of the effects of ozone on the modification of amino acid residues in glutamine synthetase and bovine serum albumin. *J Biol Chem* 271:4177–4182. <http://dx.doi.org/10.1074/jbc.271.8.4177>.
46. Lasch P, Petras T, Ullrich O, Backmann J, Naumann D, Grune T. 2001. Hydrogen peroxide-induced structural alterations of RNase A. *J Biol Chem* 276:9492–9502. <http://dx.doi.org/10.1074/jbc.M008528200>.
47. Demirev PA. 2004. Enhanced specificity of bacterial spore identification by oxidation and mass spectrometry. *Rapid Commun Mass Spectrom* 18:2719–2722. <http://dx.doi.org/10.1002/rcm.1680>.
48. Callahan C, Fox K, Fox A. 2009. The small acid soluble proteins (SASP alpha and SASP beta) of *Bacillus weihenstephanensis* and *Bacillus mycoides* group 2 are the most distinct among the *Bacillus cereus* group. *Mol Cell Probes* 23:291–297. <http://dx.doi.org/10.1016/j.mcp.2009.07.003>.
49. Petersen CE, Valentine NB, Wahl KL. 2009. Characterization of microorganisms by MALDI mass spectrometry. *Methods Mol Biol* 492:367–379.
50. Schey KL. 1996. Hydrophobic proteins and peptides analyzed by matrix-assisted laser desorption/ionization. *Methods Mol Biol* 61:227–230.
51. Achtman M, Zurth K, Morelli G, Torrea G, Guisou A, Carniel E. 1999. *Yersinia pestis*, the cause of plague, is a recently emerged clone of *Yersinia pseudotuberculosis*. *Proc Natl Acad Sci U S A* 96:14043–14048. <http://dx.doi.org/10.1073/pnas.96.24.14043>.
52. Achtman M, Morelli G, Zhu P, Wirth T, Diehl I, Kusecek B, Vogler AJ, Wagner DM, Allender CJ, Easterday WR, Chenal-Francisque V, Worsham P, Thomson NR, Parkhill J, Lindler LE, Carniel E, Keim P. 2004. Microevolution and history of the plague bacillus, *Yersinia pestis*. *Proc Natl Acad Sci U S A* 101:17837–17842. <http://dx.doi.org/10.1073/pnas.0408026101>.
53. Trebesius K, Harmsen D, Rakin A, Schmelz J, Heesemann J. 1998. Development of rRNA-targeted PCR and in situ hybridization with fluorescently labelled oligonucleotides for detection of *Yersinia* species. *J Clin Microbiol* 36:2557–2564.
54. Wittwer M, Heim J, Schar M, Dewarret G, Schurch N. 2011. Tapping the potential of intact cell mass spectrometry with a combined data analytical approach applied to *Yersinia* spp.: detection, differentiation and identification of *Y. pestis*. *Syst Appl Microbiol* 34:12–19. <http://dx.doi.org/10.1016/j.syapm.2010.11.006>.
55. Castanha ER, Fox A, Fox KF. 2006. Rapid discrimination of *Bacillus anthracis* from other members of the *B. cereus* group by mass and sequence of “intact” small acid soluble proteins (SASPs) using mass spectrometry. *J Microbiol Methods* 67:230–240. <http://dx.doi.org/10.1016/j.mimet.2006.03.024>.
56. Castanha ER, Vestal M, Hattan S, Fox A, Fox KF, Dickinson D. 2007. *Bacillus cereus* strains fall into two clusters (one closely and one more distantly related) to *Bacillus anthracis* according to amino acid substitutions in small acid-soluble proteins as determined by tandem mass spectrometry. *Mol Cell Probes* 21:190–201. <http://dx.doi.org/10.1016/j.mcp.2006.11.002>.
57. Hathout Y, Demirev PA, Ho YP, Bundy JL, Ryzhov V, Sapp L, Stutler J, Jackman J, Fenselau C. 1999. Identification of *Bacillus* spores by matrix-assisted laser desorption ionization-mass spectrometry. *Appl Environ Microbiol* 65:4313–4319.
58. Hathout Y, Setlow B, Cabrera-Martinez RM, Fenselau C, Setlow P. 2003. Small, acid soluble proteins as biomarkers in mass spectrometry analysis of *Bacillus* spores. *Appl Environ Microbiol* 69:1100–1107. <http://dx.doi.org/10.1128/AEM.69.2.1100-1107.2003>.
59. Dybwad M, van der Laaken AL, Blatny JM, Paauw A. 2013. Rapid identification of *Bacillus anthracis* spores in suspicious powder samples by using matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). *Appl Environ Microbiol* 79:5372–5383. <http://dx.doi.org/10.1128/AEM.01724-13>.
60. International Air Transport Association. 2013. Dangerous goods regulations manual, 55th ed. International Air Transport Association, Montreal, Canada.
61. International Organization for Standardization. 2006. Sterilization of health care products—radiation—part 1: requirements for development, validation, and routine control of a sterilization process for medical devices. ISO 11137-1:2006. International Organization for Standardization, Geneva, Switzerland.