Anthrax and ricin mail attacks in the U.S. demonstrated the urgent need for unambiguous verification analytics specific for bacteria, spores and toxins. MALDI-TOF MS based identification offers a powerful tool to confirm preliminary on-site results. Here we present an excellent method for identification of bacteria, spores and biological toxins that can be used as biological warfare agents.

Introduction
The changing reality of asymmetric threats posed by terrorist or rogue states in the post 9/11 era, highlights the need for rapid development of effective and efficient approaches to protect military and civilian populations against chemical/biological weapons (CBW). Particularly, this new danger is best reflected by the anthrax findings in the U.S. postal system after 9/11 and the ricin mailings in 2003 and 2013.

*Bacillus anthracis*, the causative agent of anthrax, is a pathogen of livestock and, occasionally, of humans, that persists outside of an infected host as an inert spore. The symptoms after infection of a susceptible species are severe and vary based on the kind of uptake, but can include hemorrhage, necrosis, edema, and eventually death.
In fall 2001, at least five envelopes containing powdery forms of highly concentrated $B.\ anthracis$ spore preparations ($10^{18} - 10^{13}$ colony forming units/g) were mailed through the U.S. Postal Service [1].

The glycoprotein ricin is a toxic lectin present in seeds of $Ricinus\ communis$, commonly known as the castor oil plant. Ricin can be produced in large quantities from these seeds without sophisticated or expensive technologies. Ricin is a highly toxic protein inhibiting protein synthesis, with a typical human LD50 of 3-30 µg/kg body weight by inhalation or ingestion, respectively [2]. Currently, there is no specific medicine available to treat ricin exposure.

Due to the inherent limitations of on-site detection methodologies (PCR- and immunoassay-based techniques), deployment of an independent technology is required for unambiguous verification of biological warfare agents (BWAs) in a suspicious sample. Here we describe a general solution for verification analysis of samples for the presence of BWAs using MALDI-TOF MS (Matrix-Assisted Laser Desorption/Ionization-Time-Of-Flight Mass Spectrometry).

**Experimental**

**Bacteria, spore preparation and toxins**

$Bacillus\ weihenstephanensis\ DSM\ 11821$, $B.\ thuringiensis\ DSM\ 2046$, $B.\ pseudomycoides\ DSM\ 12442$, $B.\ mycoides\ DSM\ 2048$ and $B.\ cereus\ DSM\ 31$ were obtained from DSMZ (Braunschweig, Germany). $Bacillus$ spores were produced in a modified G medium according to Hornstra et al. [3]. Preparation of inactivated $B.\ anthracis$ spores were kindly provided by the Bayerisches Landesamt für Gesundheit und Lebensmittelsicherheit (Oberschleißheim, Germany).

Different serotypes of botulinum neurotoxins (BoNTs) were obtained from Metabiologics, Inc. (Madison, WI, USA), whereas staphylococcal enterotoxin A (SEA), SEB and various plant lectins were obtained from Sigma-Aldrich (Steinheim, Germany). Purified ricin and other SEs were purchased from Toxin Technology, Inc. (Sarasota, FL, USA). Seeds of $Ricinus\ communis$ were obtained from Sandeman Seeds (Lalongue, France). Ricin was extracted from $Ricinus\ communis$ seeds using the acetone precipitation method according to Colburn et al. [4]. Toxin detection was performed with 200 µL crude extract.

**Immobilization of antibodies on paramagnetic beads**

100 µg toxin-specific antibodies (anti-SEB, anti-ricin, anti-BoNT/A, anti-BoNT/B, anti-BoNT/C, anti-BoNT/D, anti-BoNT/E, anti-BoNT/F; antibodies were kindly supplied by Dr. B. Dorner, Robert Koch-Institute) were immobilized on 5 mg M-280 tosylactivated paramagnetic Dynabeads® (Life Technologies, Darmstadt, Germany) according to the protocol of the manufacturer.

After the coupling procedure, antibody-coupled beads were adjusted with PBS containing 0.02% (v/v) Tween®-20 (PBS-T) to a concentration of 20 mg mL⁻¹.

**Sample preparation for MALDI-TOF MS analysis**

For spore analysis about 5 mg of biological material or pelleted spores (> 10⁶ spores), respectively, were inactivated in 50 µL 80% (v/v) TFA. The samples were incubated for 30 min at 25°C. After incubation, 150 µL distilled water and 200 µL acetonitrile were added to the sample. The mixture was vortexed thoroughly and subsequently centrifuged at 13,000 rpm for 3 min. An aliquot of the supernatant (1 µL) was spotted onto a ground steel target plate, air dried and overlaid with 1 µL α-cyano-4-hydroxycinnamic acid (10 mg mL⁻¹ in 50% acetonitrile/2.5% TFA). The α-cyano-4-hydroxycinnamic acid is used as a matrix for analysis of peptides by MALDI-TOF MS.

In case of toxin identification, different amounts of toxins were added to 5-10 mg powder sample re-suspended in 200 µL PBS-T buffer and filtrated through a 0.8 µm syringe filter (Whatman, UK).

The toxin containing filtrate was incubated for 1 h at 25°C under rotation in presence of antibody-coupled magnetic beads mixture (8 µL per antibody-bead conjugate). The supernatant was removed, and the beads were washed twice with 200 µL PBS-T, twice with 200 µL PBS and two more times with 200 µL distilled water. Elution of the corresponding toxin was carried out with 20 µL of 0.1% (v/v) TFA, 5% (v/v) 2,2,2-Trifluoroethanol in distilled water for 10 min at 25°C. The supernatant was transferred to a new tube and neutralized with 7 µL of 400 mM (NH₄)₂CO₃. After reduction of disulfide bonds in presence of 5 mM Dithiothreitol at 95°C and 800 rpm for 10 min and alkylation of free thiols in presence of 10 mM 2-iodoacetamide for 30 min at 25°C in the dark, enzymatic digestion was performed at 37°C and 800 rpm for 4 hours using a trypsin-to-substrate ratio of 1:20. After stopping the digestion reaction by adding TFA to a final concentration of 2% (v/v), the mixture was desalted with ZipTip® C18 (Millipore, Billerica, MA, USA), according to the manufacturer’s protocol. An aliquot of 1 µL ZipTip eluate was spotted onto a ground steel target and covered with 1 µL of saturated solution of α-cyano-4-hydroxycinnamic acid in 30:70 (v/v) acetonitrile:0.1% TFA in distilled water.

**MALDI-TOF MS analysis**

Mass spectra of bacteria, spores and toxins were acquired using a microflex™ LRF mass spectrometer (Bruker Daltonics) in the positive ion mode. For MALDI-TOF MS analysis of bacteria and spores, spectra were acquired in the linear mode within the mass range from 2,000 to 20,000 Da, and for the trypsinized toxins, spectra were achieved in the reflector mode within the mass range from 800-4,000 Da.
The MALDI Biotyper™ 3.1 software including the Bruker and SR taxonomy (containing security relevant bacteria species such as*B. anthracis, Francisella tularensis or Yersinia pestis*) was used to process and identify raw mass spectra for bacteria identification.

Results

MALDI-TOF MS fingerprinting, combined with a library of reference spectra (MALDI Biotyper library), allowing dedicated pattern matching, has been found to be excellent for robust identification of bacteria on the genus and mostly on the species level [5]. Because of the inherent ability of MALDI-TOF MS to detect the unequivocal masses of various biomolecules such as proteins and peptides, a reliable identification of BWAs can be achieved. The straightforward workflow for MALDI-TOF MS-based verification analytics of bioterrorism-relevant bacteria, spores and toxins is depicted in Fig. 1.

Identification of bioterrorism-relevant bacteria

Recently, the ability to discriminate closely related *F. tularensis* subspecies exhibiting different virulence was demonstrated [6]. A preceding culture step is mandatory for MALDI-TOF MS-based identification of bacteria. The majority of the mass signals detected from bacterial protein extracts are derived from conserved ribosomal or other abundant house-keeping proteins. Figure 2 shows the MALDI Biotyper software exemplarily identifying an unknown sample using the SR database containing all security-relevant bacteria. In this example, *Brucella melitensis* was identified after cultivation.

Identification of bioterrorism-relevant spores

In the past, there were several bioterrorism attacks using powder substances (so called “white powder attacks”). Particularly, spore-forming *B. anthracis* bacteria are considered as suitable BWAs for terrorism powder attacks due to their stability and ease of preparation. Because of the estimated high spore content in powder samples [1], in contrast to bacteria identification, the direct analysis of a powder sample without a cultivation step is possible. In case of the identification of *B. anthracis* spores, discrimination of *B. anthracis* from closely related members of the *B. cereus* group is a challenging task because of their close phylogenetic relationship. Small, acid-soluble proteins (SASPs) were found to be biomarkers for spore differentiation/identification by MS [7]. As shown in Fig. 3, SASPs could be extracted from spores after inactivation with 80% TFA and detected by MALDI-TOF MS clearly distinguishing closely related *Bacillus* strains. Spore spectra were run against the Bruker MALDI Biotyper database (> 4,600 different entries), but no matches with common pathogens exhibiting a reliable score could be observed.

Figure 1: General workflow for identification and verification of potential biological warfare agents (bacteria, spores or toxins) in suspicious samples using MALDI-TOF MS.
Figure 2: Typical identification result using MALDI Biotyper software.

Figure 3: Mass spectra (pseudo gel view) of spores from different species of the genus *Bacillus*: *B. anthracis* (A), *B. weihenstephanensis* (B), *B. thuringiensis* (C), *B. pseudomyoides* (D), *B. mycoides* (E) and *B. cereus* (F). The red arrows indicate the presence of SASPs.
Analysis of bioterrorism-relevant toxins

For MALDI-TOF MS-based toxin analysis, powder samples were re-suspended in PBS-T buffer and an immuno-magnetic separation step was applied to concentrate and separate biological toxins from powder matrix compounds [8]. After tryptic digestion and desalting of the reaction mixture, a peptide mass fingerprint (PMF) of the sample, specific for the corresponding bioterrorism-relevant toxin was generated. As shown in Fig. 4 BoNT/A, /B, /E, /F, ricin and SEB can be differentiated based on their PMFs. Furthermore, even different serotypes of BoNTs can be distinguished clearly based on their PMFs.

Analogous to the MALDI Biotype approach, a software-based identification of biological toxins is feasible. Trials were conducted using immunomagnetic separation procedure to ricin added to bentonite, to milk powder or to crude extract of Ricinus communis seeds. As shown in Fig. 5 MALDI-TOF MS allows unambiguous identification of ricin even in such complex matrices as these, as well as differentiation between ricin, agglutinin and other closely related plant lectins.

**Figure 4:** Mass spectra of tryptic digests of different biological toxins (BoNT/A, BoNT/B, BoNT/E, BoNT/F, ricin and SEB).

**Figure 5:** MALDI-TOF mass spectra of immunocaptured ricin (10 pmol) from PBS (B), bentonite (C), milk powder (D) and crude extract of Ricinus communis (E). As a positive control, the PMF of ricin was acquired without immuno-magnetic separation (A). Several characteristic ricin peaks are highlighted in grey.
**Conclusion**

MALDI-TOF MS fingerprinting allows unambiguous identification of bacteria, spores and biological toxins in suspicious bioterrorism-related samples within less than 24 hours using a single instrument. MALDI-TOF MS can differentiate serious bioterrorism attacks from harmless incidents. This differentiation is of utmost importance for rapid initiation of appropriate countermeasures, minimizing health risk and collateral damage.

A transportation protection mount permits the MALDI-TOF mass spectrometer to be used for both static and mobile laboratory installations and meet a wide variety of potential applications.

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**References**


