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# Interlaboratory Comparison of Intact-Cell Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry Results for Identification and Differentiation of *Brucella* spp.

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**Classical microbiological diagnosis of human brucellosis is time-consuming, hazardous, and subject to variable interpretation. Intact-cell matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) was evaluated for the routine identification of *Brucella* spp. Analysis of mass peak patterns allowed accurate identification to the genus level. However, statistical models based on peak intensities were needed for definite species differentiation. Interlaboratory comparison confirmed the reproducibility of the results.**

Brucellosis is a major zoonotic disease with 500,000 human cases reported worldwide annually (1). The genus *Brucella* comprises six classical species, i.e., *Brucella abortus* (biovars 1 to 6 and 9), *B. melitensis* (biovars 1 to 3), *B. suis* (biovars 1 to 5), *B. canis*, *B. ovis*, and *B. neotomae* (2), and four newly described species, i.e., *B. pinnipedialis*, *B. ceti*, *B. microti*, and *B. inopinata* (3–5). The isolation of "atypical" strains seems to increase (6–11) probably because of the higher discriminatory power of up-to-date laboratory methods and more comprehensive surveillance strategies.

Although hazardous, time-consuming, labor-intensive, and expensive, the isolation of bacteria from blood cultures and identification by classical microbiological tube testing are the current gold standards in the diagnosis of human brucellosis (12, 13). Since brucellosis is one of the most frequent laboratory-acquired infections (14), the prompt identification of a *Brucella* isolate is crucial to protect the staff members of clinical microbiology laboratories. Furthermore, brucellae are classified as category B bioterrorism agents by the Centers for Disease Control and Prevention (http://www.bt.cdc.gov/agent/agentlist-category.asp) and early detection is essential to establish effective countermeasures after an intentional release. Intact-cell matrix-assisted laser desorption ionization–time of flight mass spectrometry (ICMS) can provide an immediate tentative diagnosis based on inactivated bacterial cultures, minimizing the risk of laboratory-acquired infection and reducing the time to the treatment of affected patients (15, 16). Furthermore, appropriate sample preparation can inactivate highly infectious agents (17), simplifying the exchange of specimens.

In 1996, ICMS was used for the first time to analyze potential bioterrorism agents (18). Several years elapsed before Ferreira and colleagues (16) demonstrated its usefulness for the identification of *Brucella* isolates to the genus level. As brucellae are very closely related bacteria, with genome homologies of >90% among the members of the genus (19), the development of laboratory methods for isolate discrimination to the species and biovar levels is a challenge. Nevertheless, Lista and colleagues were able to differentiate *Brucella* species by using ICMS (15); however, primary identification to the species level was based on multilocus variable-number tandem-repeat analysis (MLVA) so that taxonomic designation remained questionable.

The aims of this study were to evaluate the interlaboratory reproducibility and stability of ICMS for the identification of brucellae to the species and biovar levels and to test the routine use of this method in clinical microbiology laboratories.

A comprehensive collection of 104 field isolates and 33 reference and vaccine strains comprising all of the currently known species and biovars of the genus *Brucella* was studied (see Table S1 in the supplemental material). The field isolates used were obtained from various geographic regions and different hosts to ensure adequate diversity. *Brucella* spp. were cultured on blood agar for 72 h at 37°C with or without 5 to 10% $CO_2$ and characterized by classical microbiological methods (13). For ICMS, proteins were extracted by the ethanol-formic acid preparation procedure (21), which reliably killed the bacteria. Mass spectra were acquired with an Ultraflex I (laboratory A; Friedrich-Loeffler-Institut, Greifswald-Insel Riems, Germany) and/or a microflex LT mass spectrometer (laboratory B; Bruker Daltonik GmbH, Bremen, Germany). MALDI Biotyper software, version 2.0 (Bruker Daltonik, Bremen, Germany), was used to create a reference database of "main spectra projections" (MSP) comprising all of the *Brucella* isolates processed in laboratory A (see Table S1) (21). The library will be made available upon request. The extent of its distribution will depend on the legal regulations of handling data on these highly pathogenic, potentially bioterroristic pathogens. At least 20 single spectra were selected from each specimen to generate MSP, containing averaged masses, averaged intensities, and statistics for

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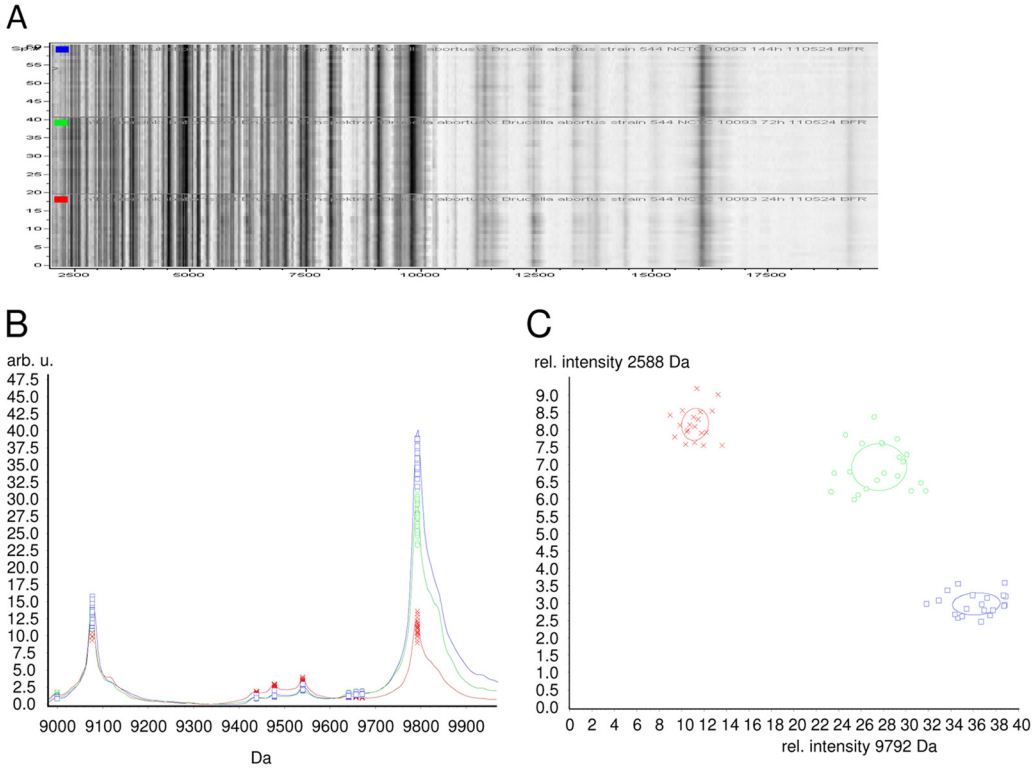


**FIG 1** Comparison of spectra originating from *B. abortus* biovar 1 (strain 544) grown for 24, 72, and 144 h. (A) Gel view of spectra obtained from *B. abortus* biovar 1 (strain 544) after 24 (red), 72 (green), and 144 (blue) h of cultivation. Although the qualitative mass pattern remained stable over time, marked quantitative changes (shown in panels B and C) were observed. (B) The intensities of various peaks (e.g., that at 9,792 Da) changed significantly over time, while others, such as that at 9,008 Da, did not (peak intensities in arbitrary units [arb. u.]). (C) Scatter plot representing peak intensities at 2,588 and 9,792 Da. The graph shows that spectra generated at different time points can be well separated by relative (rel.) peak intensities.

the reproducibility of characteristic peaks. For comparison of two spectra, MALDI Biotyper calculates MSP-based similarity scores ranging from 0 (no similarity) to 3 (identity).

**Kinetic stability of mass patterns.** For routine applications, analysis of the same sample in different laboratories must lead to the same identification result. ICMS-based identification of bacteria to the species level has already proved to be independent of the device used (23), and interlaboratory results were highly reproducible (25). However, the discriminating peaks of *Brucella* spp. described by Ferreira and colleagues (16) were not always present in the spectra generated in this study, although the technical equipment and mass spectrometry methods used were comparable. Different growth conditions (chocolate agar for 48 h [16] versus blood agar for 72 h [this study]) may have resulted in variable profiles. A significant influence of the culture medium used was excluded (16), but the age of the culture proved to be crucial. *B. abortus* biovar 1 (strain 544), *B. melitensis* biovar 1 (strain 16M), and *B. suis* biovar 1 (strain 1330) in different growth phases (after 24, 48, 72, 96, 120, and 144 h of cultivation) showed considerable changes in peak intensities (Fig. 1). Hence, culture conditions have to be strictly defined to generate reproducible spectra.

**Identification by MALDI Biotyper.** Analysis of the spectrum-based Euclidean distances between *Brucella* species and biovars (Fig. 2) on the basis of MSP generated with MALDI Biotyper (version 1.1) and the statistical programming language R, version 2.13 (24), revealed a number of disagreements between ICMS and classical taxonomy based on host specificity and phenotypic traits. However, ICMS correlated with MLVA typing and metabolic biotyping data (15, 26), questioning the raison d'être of the currently used classification scheme. Especially, *B. abortus* and *B. melitensis* strains displayed highly similar spectra so that unambiguous discrimination was difficult. *B. canis*, *B. ovis*, and *B. suis* biovars 3 and 4 also showed a continuum of phenotypes that could be only partially resolved by principal-component analysis (data not shown). In contrast, *B. suis* formed a multicentric pattern composed of well-separated clusters of biovars 1, 2, 3/4, and 5.

Nevertheless, the identification results provided by the algorithm of the MALDI Biotyper software were quite reliable despite the close relatedness of the spectra. At the species level, a total of 92% of the bacterial samples were correctly identified (90% in laboratory A and 95% in laboratory B). In four cases, misidentifications occurred in the *B. abortus*/*B. melitensis* group. *B. ceti* was misdiagnosed as *B. pinnipedialis* in three cases and as *B. canis* in another one. *B. suis* was misidentified seven times, five times as *B. canis*, once as *B. abortus*, and once as *B. melitensis*. Incorrect biovar assignments were frequently found in *B. abortus* (28/41) and *B. melitensis* (25/52). In contrast, all 43 correctly identified *B. suis* strains were accurately assigned to their respective biovars.

**Statistical models.** A set of marker masses specific for every single species within the genus *Brucella* could not be ascertained. To resolve misidentifications reported by MALDI Biotyper and to finally assign strains with borderline scores to the correct species, ambiguous spectra were classified by the application of multivariate statistical analysis. Statistical models were calculated on the

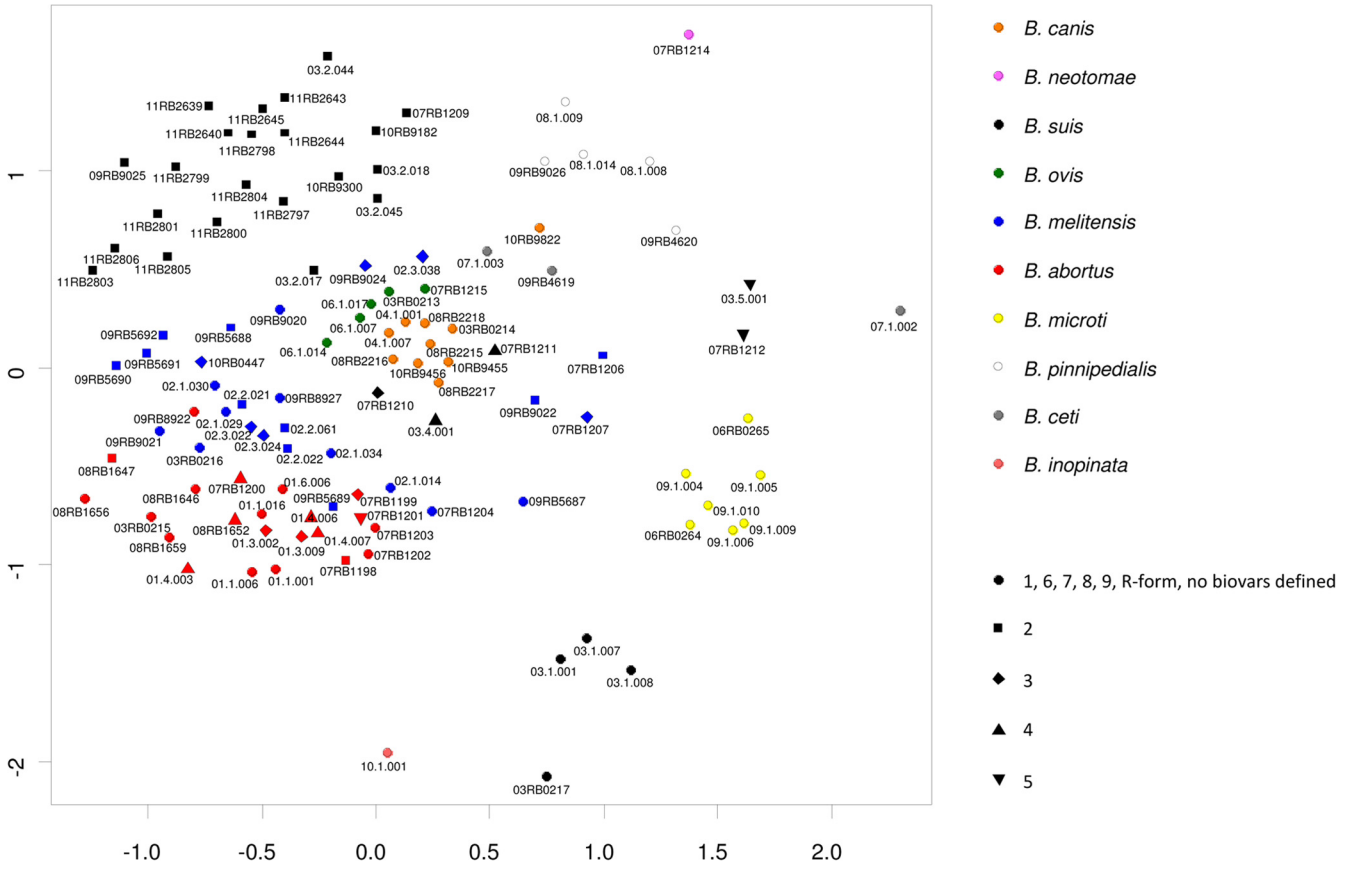


**FIG 2** Distance relationships of mass spectra deriving from *Brucella* species. Euclidian distances between reference spectra of *Brucella* isolates were calculated on the basis of mass alignment (20, 22) and are depicted as a Sammon map (27) that shows the highly dimensional distances in a two-dimensional plane with minimized distortion. The colors represent *Brucella* species, and the symbols represent biovars. Note that some species and biovars (e.g., *B. microti*, *B. suis* biovar 1) form compact and spherical subgroups whereas others (e.g., *B. melitensis*, *B. suis*) display multicentric and broader distributions.

basis of four randomly selected spectra originating from laboratory A by using the Support Vector Machine algorithm of the ClinProTools (CPT) software (version 2.2; Bruker Daltonik). Models were externally validated with the remaining spectra that were recorded for MSP generation in laboratory A but not used in the model and with the respective spectra from laboratory B.

Considering the sporadic erroneous identification results produced by MALDI Biotyper, the partial overlaps of various species

**TABLE 1** Summary of the classification results obtained with MALDI Biotyper and CPT software

| Sample | MALDI Biotyper query result | | | |
|---|---|---|---|---|
| | No. correct/total | No. incorrect/total[a] | Misdiagnosis[b] | CPT classification |
| *B. abortus* | 41/44 | 3/44 | *B. melitensis* | NA[c] |
| *B. canis* | 21/21 | | | |
| *B. ceti* | 5/9 | 3/9 | *B. pinnipedialis* | *B. ceti* vs *B. pinnipedialis* |
| | | 1/9 | *B. canis* | *B. canis* vs *B. ceti* |
| *B. melitensis* | 52/53 | 1/53 | *B. abortus* | NA |
| *B. microti* | 12/12 | | | |
| *B. ovis* | 10/10 | | | |
| *B. pinnipedialis* | 8/8 | | | |
| *B. suis* biovar 1 | 9/10 | 1/10 | *B. canis* | *B. canis* vs *B. suis* biovar 1 |
| *B. suis* biovar 2 | 29/29 | | | |
| *B. suis* biovar 3 or 4 | 2/6 | 4/6 | *B. canis* | *B. canis* vs *B. suis* biovar 3/4 |
| *B. suis* biovar 5 | 3/5 | 1/5 | *B. abortus* | *B. suis* biovar 5 vs *B. abortus*/*melitensis* |
| | | 1/5 | *B. melitensis* | *B. suis* biovar 5 vs *B. abortus*/*melitensis* |

[a] For *Brucella* species and for the biovars of *B. suis* of which not all representatives were correctly identified by MALDI Biotyper software, statistical models were developed that allowed unambiguous identification. For the parameters of the models (CPT classification column), see Table S2 in the supplemental material.
[b] The misdiagnosis column contains the top hits of the MALDI Biotyper query in cases of incorrect species identification.
[c] NA indicates that no models could be deduced with CPT software that performed better than MALDI Biotyper.

and biovars in the Sammon map (Fig. 2), and the small distances of score values obtained for representatives of different species, models separating the following classes were constructed: *B. canis* versus *B. ovis*, *B. canis* versus *B. suis* biovar 1, *B. canis* versus *B. suis* biovars 3 and 4, *B. ceti* versus *B. pinnipedialis*, and *B. suis* biovar 5 versus *B. abortus* and *B. melitensis*. For details of the statistical models used, see Table S2 in the supplemental material. In combination with MALDI Biotyper results, this approach correctly classified all of the *Brucella* species (Table 1) and reliably identified the biovars of *B. suis* isolates as far as a statistical model has been established.

In summary, for routine clinical microbiology, ICMS is a convenient and efficient molecular analytic tool for the first-line diagnosis of brucellosis, provided that bacterial culture conditions are well controlled, standardized sample preparation protocols are followed, and databases including high-quality reference spectra are carefully maintained (21). Clustering of spectra is not in line with classical taxonomy, but identification of all *Brucella* isolates to the species level with very high sensitivity and specificity was possible. Statistical analysis was validated with independent spectra from a second laboratory and proved to be robust.

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Markus Kostrzewa and Markus Timke have potential conflicts of interest, as they are both employees of Bruker Daltonik GmbH, which produces the matrix-assisted laser desorption ionization–time of flight mass spectrometry system used in this study, as well as the MALDI Biotyper and the CPT software packages. The rest of us have no competing interests.

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