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Evaluation of MALDI-TOF MS and an expanded custom reference spectra database for the identification and differentiation of *Taylorella equigenitalis* and *Taylorella asinigenitalis*

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ABSTRACT

Misidentification between *Taylorella equigenitalis*, the causative agent of contagious equine metritis (CEM), and *Taylorella asinigenitalis* is observed by the gold standard culture method. The performance of matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF MS) for *Taylorella* species identification was evaluated using 85 *T. equigenitalis* and 28 *T. asinigenitalis* strains selected on the basis of multilocus sequence typing data. Seven of the *T. equigenitalis* and 9 of the *T. asinigenitalis* strains were used to generate in-house reference spectra to expand the existing commercial Bruker database. Two bacterial incubation times and 3 different sample preparation procedures were compared. Overall, we demonstrated the usefulness of MALDI-TOF MS as a differential diagnostic tool for CEM; however, commercial spectra databases should be expanded with *T. asinigenitalis* reference spectra to achieve the expected performance. Moreover, direct spotting of 48-h colonies was not only the most efficient protocol but also the easiest to implement in a clinical setting.
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1. Introduction

The *Taylorella* genus classified in the Alcaligenaceae family is divided into 2 species, *Taylorella equigenitalis* (Sugimoto et al., 1983) and *Taylorella asinigenitalis* (Jang et al., 2001). *T. equigenitalis* is reported from the genital tract of horses and rarely of donkeys, and causes contagious equine metritis (CEM), a sexually transmitted disease, included in the World Organisation for Animal Health (OIE) list of notifiable terrestrial and aquatic animal diseases (http://www.oie.int/en/animal-health-in-the-world/oie-listed-diseases-2019/). CEM was first reported in 1977 in the United Kingdom and Ireland from Thoroughbred horses (Crowhurst, 1977; Timoney et al., 1977) and is currently detected worldwide in various horse breeds. The high contagiousness and substantial economic losses associated with CEM led the Horserace Betting Levy Board to develop a Code of Practice for swabbing mares and stallions and for horse breeding disease management (http://codes.hblb.org.uk/) (Allen and Wilsher, 2018). *T. asinigenitalis* is reported from the genital tract of donkeys and less frequently horses but is not considered to be pathogenic. The gold standard in CEM diagnosis is the culture method (OIE, 2012), but misidentification with *T. asinigenitalis* is observed since phenotypic tests (based on catalase, oxidase, and phosphatase activities, and reactivity with *T. equigenitalis*–specific antiserum) are not sufficiently discriminating. Alternative methods such as indirect immunofluorescence (IIF) (Breuil et al., 2010), polymerase chain reaction (PCR) (Breuil et al., 2011) and 16S rDNA sequencing are still needed.

Matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF MS) has emerged as a new technology to replace conventional phenotypic tests for species identification and is increasingly used in many diagnostic laboratories worldwide. Despite i) the high initial cost of the MALDI-TOF equipment and ii) the accessibility to an exhaustive spectral database for the bacterial species identification, MALDI-TOF MS identification is rapid, accurate, and less expensive in routine use than molecular and immunological detection methods (Patel, 2015; Singhal et al., 2015). The results of identification using MALDI TOF MS depend on the microorganism, sample preparation procedure, the matrix used for the reaction, and mostly the spectra present in the database used to obtain the identification. Within this context, we aimed to evaluate the performance of MALDI-TOF MS for the identification of *Taylorella* species. Two different bacterial incubation times and 3 different sample preparation procedures were compared. Sixteen in-

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house *Taylorella* reference spectra were generated during this study to expand the existing commercial Bruker database to 10 reference spectra per *Taylorella* species.

2. Materials and methods

2.1. Bacterial strains, their identification, and selection

A total of 113 *Taylorella* species strains were analyzed in this study: 110 isolates and 3 reference strains from the Collection Institut Pasteur (CIP, France): *T. equigenitalis* CIP 79.7^T (= ATCC 35865 = NCTC11184), *T. equigenitalis* CIP 79.44 (= NCTC 11225), and *T. asinigenitalis* CIP 107673^T (= UCD-1 = ATCC 700933). Selection of isolates was based on multilocus sequence typing (MLST) data previously obtained according to the Duquesne et al. (2013) MLST scheme developed for both *Taylorella* species and available in the *Taylorella* MLST database (https://pubmlst.org/taylorella/). Isolates were 83 *T. equigenitalis* strains from 71 horses and 2 donkeys (at least 35 males and 22 females) located in France (77%), Belgium, Poland, Switzerland, the United Arab Emirates, and unknown geographical locations, and 27 *T. asinigenitalis* strains from 6 horses and 11 donkeys (at least 14 males and 1 female) located in France (82%), Belgium, Sweden, and Switzerland. They were identified at the institutions of origin at least using the gold standard culture method according to OIE (OIE, 2012) or national culturing instructions for the detection of CEM, e.g., AFNOR standard NF U47-108 (AFNOR, 2012) for strains isolated in France. On receipt of isolates at the Dozulé Laboratory for Equine Diseases (ANSES, France), which is appointed as the French reference laboratory for CEM and the European Union reference laboratory for equine diseases other than African horse sickness (https://eurl-equinediseases.anses.fr/), the *Taylorella* species was confirmed using a *T. equigenitalis*–specific IIF technique (Breuil et al., 2010) and *Taylorella* species–specific PCRs (Breuil et al., 2011; Duquesne et al., 2007). Isolates and reference strains were maintained using cryobeads at −80 °C.

2.2. MALDI-TOF MS

All isolates and reference strains were subcultured on ready-to-use chocolate agar with polyvitamin supplement (BioMerieux, France) for 48 h and 72 h at 37 ± 2 °C in 7% (*v*/v) CO_2 in air and identified using a Microflex LT mass spectrometer (Bruker Daltonics, Germany) with MALDI BioTyper and FlexControl V3.0 software. Three different sample preparation procedures were tested on 3 colonies per strain and per sample preparation procedure: i) direct transfer (spotting) of the colony onto a target plate, ii) formic acid overlay method that consists in depositing 1 μL of formic acid on direct colony spotting, and total protein extraction (only for *T. asinigenitalis* species) using the acetonitrile/formic acid protocol, according to the Bruker Daltonics manufacturer's recommendations. For the total protein extraction, one colony was suspended in 300 μL of ultrapure water, vortexed, and added to 900 μL of ethanol. After centrifugation at 16,000 ×*g* for 2 min, the supernatant was discarded, and the pellet was dried at room temperature without a secondary centrifugation step. The dried pellet was mixed with 10 μL of 70% acid formic solution and then with 10 μL of acetonitrile solution. After centrifugation at 16,000 ×*g* for 2 min, 1 μL of supernatant was spotted onto a target plate in 3 replicates. For each protocol tested, dried deposits were overlaid with 1 μL of 10 mg/mL of α-cyano-4-hydroxycinnamic acid (HCCA) matrix solution (Bruker Daltonics, Germany). The MALDI Biotyper system was calibrated with a Bruker Bacterial Test Standard (*Escherichia coli* DH5α), and the spectra for proteins with mass between 2000 to 20,000 Da were obtained and matched with the Bruker V7.0.0 database composed of 8223 mass spectrometry profiles (MSPs), including 3 *T. equigenitalis* MSPs from strains DSM 10668^T, GDD 39, and GDD 40, and 1 *T. asinigenitalis* MSP from the CIP 107673^T strain.

The implementation of in-house reference MSPs was done using 7 *T. equigenitalis* strains and 9 *T. asinigenitalis* strains to expand the Bruker database. MSPs were generated using 26–32 spectra from total protein extracts spotted onto a target plate in 8 replicates, and each spot was analyzed 4 times. All raw spectra were done with a laser frequency of 60 Hz, an acceleration voltage of 20 kV, and extraction delay time of 120 ns and analyzed according to the established MSP protocol of the Maldi Biotyper® V1.1 to remove suboptimal spectra. Preprocessing of the MSP was done using the Biotyper MSP creation standard method (Bruker Daltonics, Germany) with a default parameter set for the baseline correction (multipolygon with search window 5 and number of runs 2) and the spectral smoothing (Savitzky–Golay with frame size 25). For each strain, 2 MSPs were generated, respecting a repeatability condition (same culture, same analysis).

The cutoff MALDI TOF Biotyper classification scores for identification were those recommended by the manufacturer: <1.70, no reliable identification; ≤1.99 and ≥1.70, high genus-level identification; ≥2.00, high species-level identification. The highest score among the replicates of a strain was considered to define the number of unidentified and correctly identified strains. The performance of MALDI TOF MS was evaluated in terms of its typeability and accuracy; typeability refers to the ability of MALDI TOF MS to assign a species name to a sample with a score ≥1.70, and accuracy is the ability of MALDI TOF MS to obtain the correct species name for a sample.

2.3. Retrospective analysis of MALDI-TOF MS results from a veterinary diagnostic laboratory

The veterinary diagnostic laboratory LABOCEA22 (Ploufragan, France) implemented MALDI-TOF MS in clinical microbiology diagnosis almost 10 years ago (2009–2010). Between 2011 and 2017, microbiological investigations were performed on 4920 genital samples, including 2330 from equine species that were all CEM-negatives by the culture method according to AFNOR Standard NF U47-108 (AFNOR, 2012). Isolated bacterial colonies from these microbiological investigations were identified by MALDI-TOF MS using a Microflex LT mass spectrometer with MALDI BioTyper and FlexControl software (Bruker Daltonics, Germany). Colonies were directly spotted and overlaid with 1 μL HCCA matrix solution (Bruker Daltonics, Germany) according to the manufacturer's recommendations. The bacterial test standard (Bruker) was used for instrument calibration. The cutoff scores for identification were those recommended by the manufacturer.

3. Results

In all, 113 *Taylorella* species isolates and reference strains were selected based on MLST data to evaluate the identification and differentiation of *T. equigenitalis* ($n = 85$) and *T. asinigenitalis* ($n = 28$) by MALDI-TOF MS. The MLST results of these 113 strains yielded a total of 46 sequence types (STs) distributed in 31 STs for *T. equigenitalis* (0.36 STs/strain) and 15 STs for *T. asinigenitalis* (0.54 STs/strain). Moreover, all strains were previously identified by the gold standard culture method. Each *T. equigenitalis* strain was *T. equigenitalis*–specific IIF positive, *T. equigenitalis*–specific PCR positive, and *T. asinigenitalis*–specific PCR negative, and conversely, each *T. asinigenitalis* strain was *T. equigenitalis*–specific IIF negative, *T. equigenitalis*–specific PCR negative, and *T. asinigenitalis*–specific PCR positive. Among these strains, 7 *T. equigenitalis* and 9 *T. asinigenitalis* were selected based on MLST data to generate 16 in-house *Taylorella* reference spectra to expand the existing commercial Bruker database to 10 reference spectra per *Taylorella* species.

Colony diameter reached 1.0–1.5 mm after an incubation time of 48 h for *T. equigenitalis* and 72 h for *T. asinigenitalis* on chocolate agar with polyvitamin supplement at 37 ± 2 °C in 7% (*v*/v) CO_2 in air. In order to carry out a MALDI-TOF MS analysis as soon as the *Taylorella* species colonies were visualized, the 48-h and 72-h incubation times

were compared. Three isolated 48-h and 72-h colonies per strain and per sample preparation procedure were processed by direct spotting and formic acid extraction, and 3 isolated 48-h and 72-h colonies per *T. asinigenitalis* strain were also processed by total protein extraction.

MALDI-TOF MS identifications are presented in Table 1. The MALDI-TOF MS system accurately differentiated both species, *T. equigenitalis* and *T. asinigenitalis*. With the existing Bruker database, the results showed typeability of 100% for *T. equigenitalis* and 93–100% for *T. asinigenitalis*, and achieved accuracy of 100%. Overall, 85 (100%) *T. equigenitalis* strains were identified in all conditions tested with a species-level identification of 98.8–100%. Additionally, 26 (93%) to 28 (100%) *T. asinigenitalis* strains were identified with a species-level identification of only 26–43% after formic acid extraction, 59–61% with direct spotting, and 75–77% after total protein extraction. With the Bruker database expanded with 7 *T. equigenitalis* and 9 *T. asinigenitalis* reference spectra (Bruker database, expanded), all results showed 100% typeability and accuracy, and all strains per conditions tested were identified with a species-level identification of 100%.

MALDI-TOF MS score distributions are depicted using violin plots (-Fig. 1). As expected, the score values were increased for *T. equigenitalis* (Fig. 1A and B) and especially *T. asinigenitalis* (Fig. 1C and D) with the expanded Bruker database (Fig. 1B and D) compared to the Bruker database (Fig. 1A and C); the score values for the 85 *T. equigenitalis* strains ranged from 1.52 to 2.54 (mean ± SD: 2.28 ± 0.10) with the Bruker database and 1.84 to 2.67 (2.43 ± 0.09) with the expanded Bruker database, while the values for the 28 *T. asinigenitalis* strains ranged from 1.33 to 2.47 (1.92 ± 0.16) with the Bruker database and 1.64 to 2.78 (2.42 ± 0.17) with the expanded Bruker database. For *T. equigenitalis*, best results were obtained when colonies were processed by direct spotting compared to formic acid extraction. Significant differences across both bacterial incubation times were observed only when colonies were processed by formic acid extraction, with better results for 72-h colonies compared to 48-h colonies. For *T. asinigenitalis*, better results with the Bruker database were obtained when colonies were processed by direct spotting and by total protein extraction compared to formic acid extraction. In contrast, no significant differences were observed across the 3 sample preparation procedures with the expanded Bruker database. However, interestingly, score distributions with total protein extraction were the most extensive at values <2.00. Concerning the bacterial incubation times, significant differences were observed only when colonies were processed by total protein extraction, with better results for 72-h colonies compared to 48-h colonies.

A retrospective analysis of MALDI-TOF MS results from a veterinary diagnostic laboratory (LABOCEA22) on isolated bacterial colonies of 2330 equine samples, which were all CEM-negatives by the culture method, showed 100% specificity: no *T. equigenitalis* and *T. asinigenitalis* identifications were observed by MALDI-TOF MS.

4. Discussion

In this study, the usefulness and accuracy of MALDI-TOF MS for the identification and differentiation of *T. equigenitalis*, the causative agent of CEM, and *T. asinigenitalis* were evaluated. With the existing commercial Bruker database, including 3 *T. equigenitalis* reference spectra and a single *T. asinigenitalis* reference spectrum, our findings revealed excellent MALDI-TOF MS performance at the species level for *T. equigenitalis* and at the genus level for *T. asinigenitalis*. The poorer results obtained with the *T. asinigenitalis* strains can be attributed to the presence of a single *T. asinigenitalis* reference spectrum included in the Bruker database that is not sufficiently representative of the species in circulation, especially in a context where the genetic diversity of *T. asinigenitalis* appears to be higher than that of *T. equigenitalis* (Duquesne et al., 2013). Nevertheless, it is important to note that no *T. asinigenitalis* were falsely interpreted even though *T. equigenitalis* is its closest phylogenetic relative, confirming the potential of MALDI TOF MS to perform correct discrimination between both *Taylorella* species. Retrospective analysis of MALDI-TOF MS results from a veterinary diagnostic laboratory, LABOCEA22, confirmed that no *T. equigenitalis* and *T. asinigenitalis* were falsely interpreted from all microbiological investigations on CEM-negative equine samples between 2011 and 2017.

Finally, the performance of MALDI-TOF MS for the identification of *T. asinigenitalis* became excellent at the species level when the Bruker database was expanded with in-house *T. asinigenitalis* reference spectra generated during this study. Importantly, the addition of the *T. equigenitalis* reference spectra simply made it possible to improve the score values that were already at species-level identification. Improvement of identification with an expanded database has been shown in various studies on different microorganisms (Christensen et al., 2012; Pérez-Sancho et al., 2018; Sogawa et al., 2012). The presence of exhaustive reference MSPs in a commercial database is a critical

Table 1
Taylorella species identification by MALDI-TOF MS against the Bruker database or Bruker database expanded with 7 *T. equigenitalis* and 9 *T. asinigenitalis* in-house reference spectra acquired during this study.

Sample preparation procedure [a]	Bacterial incubation time	No peaks found		Misidentified [c]		Unidentified [d]; score < 1.70		Correct genus [d]; score 1.70 to <2.00		Correct species [d]; score ≥ 2.00	
		TE [b]	TA [b]	TE	TA	TE	TA	TE	TA	TE	TA
Bruker database											
A	48 h	0	0	0	0	0	0	0	11	85	17
	72 h	0	0	0	0	0	1	0	11	85	16
B	48 h	0	0	0	0	0	0	1	16	84	12
	72 h	0	0	0	0	0	1	0	20	85	7
C	48 h	nt [b]	1	nt	0	nt	1	nt	6	nt	20
	72 h	nt	0	nt	0	nt	0	nt	7	nt	21
Bruker database, expanded											
A	48 h	0	0	0	0	0	0	0	0	85	28
	72 h	0	0	0	0	0	0	0	0	85	28
B	48 h	0	0	0	0	0	0	0	0	85	28
	72 h	0	0	0	0	0	0	0	0	85	28
C	48 h	nt	0	nt	0	nt	0	nt	0	nt	28
	72 h	nt	0	nt	0	nt	0	nt	0	nt	28

[a] Three sample preparation procedures were compared: A, direct spotting; B, formic acid extraction; C, total protein extraction.
[b] TE = *T. equigenitalis* ($n = 85$); TA = *T. asinigenitalis* ($n = 28$); nt = not tested.
[c] MALDI-TOF MS species is not the same as the reference (scores from 1.17 to 1.51).
[d] MALDI-TOF MS species is the same as the reference. Manufacturer's score interpretation: <1.70, no reliable identification; ≤1.99 and ≥1.70, genus-level identification; ≥2.00, species-level identification.

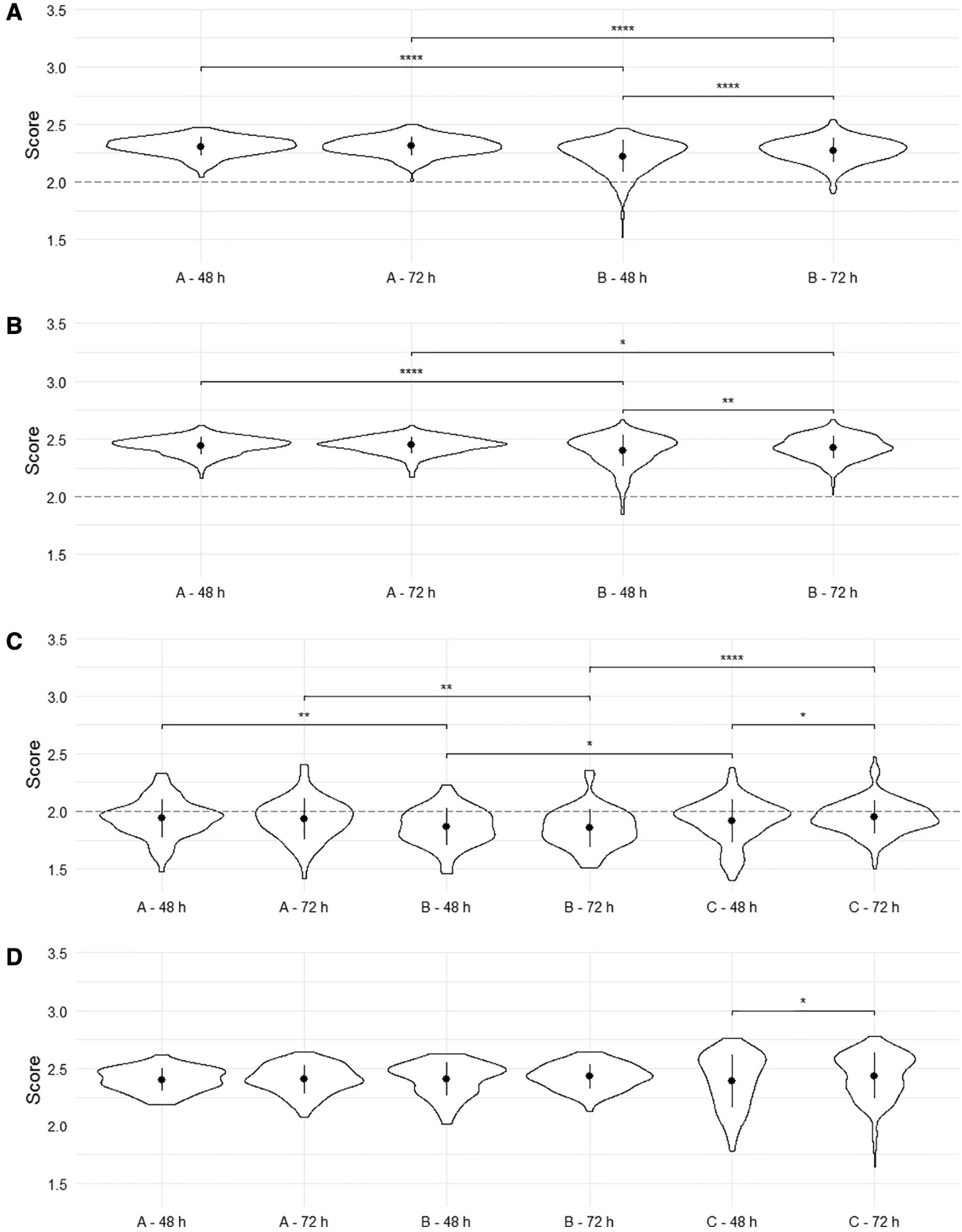

Fig. 1. Violin plots of MALDI-TOF MS scores between Bruker database, expanded or not, bacterial incubation times, and sample preparation procedures. Spectra from 85 *T. equigenitalis* strains (A, B) and spectra from 28 *T. asinigenitalis* strains (C, D) were analyzed against the Bruker database (A, C) or the Bruker database expanded with 7 *T. equigenitalis* and 9 *T. asinigenitalis* in-house reference spectra acquired during this study (B, D). Three isolated 48-h and 72-h colonies per strain and per sample preparation procedure were processed by direct spotting (A, 48 h and A, 72 h), formic acid extraction (B, 48 h and B, 72 h), and total protein extraction (C, 48 h and C, 72 h); total protein extracts were spotted in 3 replicates. Dots and error bars represent the mean values and standard deviations, respectively. Statistically significant differences ($P < 0.05$; 2-tailed Student's *t* test) are marked with asterisks (*$P < 0.05$; **$P < 0.01$; ****$P < 0.0001$).

point for this technology for microorganism identification. To meet this requirement, numerous species are needed, along with expansive and time-consuming work to obtain a validated database.

Two to 3 sample preparation procedures were compared during this study. Overall, the easier sample preparation procedure by direct colony spotting seems sufficient to obtain excellent MALDI-TOF MS performance at the species level. In all cases, *T. equigenitalis* score distributions were significantly increased by direct colony spotting. The differences between the sample preparation procedures were less marked for *T. asinigenitalis*. In fact, no impact of the sample preparation procedure was observed between i) direct spotting and total protein extraction with the existing Bruker database and ii) direct spotting, formic acid extraction, and total protein extraction with the Bruker database expanded with in-house *Taylorella* reference spectra. Nevertheless, *T. asinigenitalis* score distributions with total protein extraction were the most extensive at values <2.00, while the number of strains identified at the species level was the highest.

Two bacterial incubation times were compared during this study. Overall, the shortest bacterial incubation time (48 h compared to 72 h) was sufficient to obtain excellent MALDI-TOF MS performance at the species level. Essentially, differences between the bacterial incubation times tested were either not significant, e.g., with direct spotting for both *Taylorella* species and with formic acid extraction for *T. asinigenitalis*, or they were without any real impact on *Taylorella* species identification, e.g., with formic acid extraction for *T. equigenitalis* and with total protein extraction for *T. asinigenitalis*.

In summary, we have demonstrated that MALDI-TOF MS is currently a highly reliable tool for the species-level identification of *T. equigenitalis* and *T. asinigenitalis*. MALDI-TOF MS could be a useful and rapid differential diagnostic tool to establish the prevalence and epidemiological role of both *Taylorella* species in the clinical setting. However, existing commercial MALDI-TOF MS spectra databases should be expanded with *T. asinigenitalis* reference spectra to achieve the expected performance of *T. asinigenitalis* identification at a species level. Additionally, for *T. asinigenitalis,* which has slower growth than *T. equigenitalis*, direct spotting of 48-h colonies presented overall the most reliable and consistent identification rates, and it was the easiest protocol to implement in the clinical setting. On the basis of our findings, we propose to amend the OIE Terrestrial Manual (OIE, 2012) with the addition of MALDI-TOF MS as a differential diagnostic tool for CEM.

Competing interests

The authors declare that they have no competing interests.

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