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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1 **Running title: MALDI-TOF MS identification of *Taylorella* species**

2

3 **Evaluation of MALDI-TOF MS and an expanded custom reference spectra database for**
4 **the identification and differentiation of *Taylorella equigenitalis* and *Taylorella***
5 ***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 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 (MLST) data. Seven of the *T. equigenitalis* and nine 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 three 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.

Keywords: MALDI-TOF MS; *Taylorella equigenitalis*; *Taylorella asinigenitalis*; contagious equine metritis; infectious equine disease.

43 **1. Introduction**

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

62 Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-
63 TOF MS) has emerged as a new technology to replace conventional phenotypic tests for
64 species identification and is increasingly used in many diagnostic laboratories worldwide.
65 Despite *(i)* the high initial cost of the MALDI-TOF equipment and *(ii)* the accessibility to an
66 exhaustive spectral database for the bacterial species identification, MALDI-TOF MS
67 identification is rapid, accurate and less expensive in routine use than molecular and

68 immunological detection methods (Patel, 2015; Singhal et al., 2015). The results of
69 identification using MALDI TOF MS depend on the microorganism, sample preparation
70 procedure, the matrix used for the reaction, and mostly the spectra present in the database
71 used to obtain the identification. Within this context, we aimed to evaluate the performance of
72 MALDI-TOF MS for the identification of *Taylorella* species. Two different bacterial
73 incubation times and three different sample preparation procedures were compared. Sixteen
74 in-house *Taylorella* reference spectra were generated during this study to expand the existing
75 commercial Bruker database to 10 reference spectra per *Taylorella* species.

76

77 **2. Materials and methods**

78 *2.1. Bacterial strains, their identification and selection*

79 A total of 113 *Taylorella* species strains were analysed in this study: 110 isolates and
80 three reference strains from the Collection Institut Pasteur (CIP, France): *T. equigenitalis* CIP
81 79.7^{T} (= ATCC 35865 = NCTC11184), *T. equigenitalis* CIP 79.44 (= NCTC 11225), and *T.*
82 *asinigenitalis* CIP 107673^{T} (= UCD-1 = ATCC 700933). Selection of isolates was based on
83 MLST data previously obtained according to the Duquesne *et al*. (2013) MLST scheme
84 developed for both *Taylorella* species, and available in the *Taylorella* MLST database
85 (https://pubmlst.org/taylorella/). Isolates were 83 *T. equigenitalis* strains from 71 horses and
86 two donkeys (at least 35 males and 22 females) located in France (77%), Belgium, Poland,
87 Switzerland, the United Arab Emirates and unknown geographical locations, and 27 *T.*
88 *asinigenitalis* strains from six horses and 11 donkeys (at least 14 males and one female)
89 located in France (82%), Belgium, Sweden and Switzerland. They were identified at the
90 institutions of origin at least using the gold standard culture method according to OIE (OIE,
91 2012) or national culturing instructions for the detection of CEM, e.g. AFNOR standard NF
92 U47-108 (AFNOR, 2012) for strains isolated in France. On receipt of isolates at the Dozulé

93 Laboratory for Equine Diseases (ANSES, France), which is appointed as the French reference
94 laboratory for CEM and the European Union reference laboratory for equine diseases other
95 than African horse sickness (https://eurl-equinediseases.anses.fr/), the *Taylorella* species was
96 confirmed using a *T. equigenitalis*-specific IIF technique (Breuil et al., 2010) and *Taylorella*
97 species-specific PCRs (Duquesne et al., 2007; Breuil et al., 2011). Isolates and reference
98 strains were maintained using cryobeads at -80 °C.
99

100 *2.2. MALDI-TOF MS*

101 All isolates and reference strains were subcultured on ready-to-use chocolate agar with
102 polyvitamin supplement (BioMerieux, France) for 48 h and 72 h at 37±2°C in 7% (v/v) CO_2
103 in air, and identified using a Microflex LT mass spectrometer (Bruker Daltonics, Germany)
104 with MALDI BioTyper and FlexControl V3.0 software. Three different sample preparation
105 procedures were tested on three colonies per strain and per sample preparation procedure: *(i)*
106 direct transfer (spotting) of the colony onto a target plate, *(ii)* formic acid overlay method that
107 consists in depositing 1 µl of formic acid on direct colony spotting, and total protein
108 extraction (only for *T. asinigenitalis* species) using the acetonitrile/formic acid protocol,
109 according to the Bruker Daltonics manufacturer's recommendations. For the total protein
110 extraction, one colony was suspended in 300 µl of ultrapure water, vortexed and added to 900
111 µl of ethanol. After centrifugation at 16,000 g for 2 min, the supernatant was discarded and
112 the pellet was dried at room temperature without a secondary centrifugation step. The dried
113 pellet was mixed with 10 µl of 70% acid formic solution and then with 10 µl of acetonitrile
114 solution. After centrifugation at 16,000 g for 2 min, 1 µl of supernatant was spotted onto a
115 target plate in three replicates. For each protocol tested, dried deposits were overlaid with 1 µl
116 of 10 mg/ml of α-cyano-4-hydroxycinnamic acid (HCCA) matrix solution (Bruker Daltonics,
117 Germany). The MALDI Biotyper system was calibrated with a Bruker Bacterial Test Standard

118 (*Escherichia coli* DH5α), and the spectra for proteins with mass between 2 000 to 20 000 Da
119 were obtained and matched with the Bruker V7.0.0 database, composed of 8 223 mass
120 spectrometry profiles (MSPs), including three *T. equigenitalis* MSPs from strains DSM
121 10668^T, GDD 39 and GDD 40, and one *T. asinigenitalis* MSP from the CIP 107673^T strain.

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

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

141

142 *2.3. Retrospective analysis of MALDI-TOF MS results from a veterinary diagnostic*
143 *laboratory*

144 The veterinary diagnostic laboratory LABOCEA22 (Ploufragan, France) implemented
145 MALDI-TOF MS in clinical microbiology diagnosis almost 10 years ago (2009-2010).
146 Between 2011 and 2017, microbiological investigations were performed on 4 920 genital
147 samples, including 2 330 from equine species that were all CEM-negatives by the culture
148 method according to AFNOR Standard NF U47-108 (AFNOR, 2012). Isolated bacterial
149 colonies from these microbiological investigations were identified by MALDI-TOF MS using
150 a Microflex LT mass spectrometer with MALDI BioTyper and FlexControl software (Bruker
151 Daltonics, Germany). Colonies were directly spotted and overlaid with 1 µl HCCA matrix
152 solution (Bruker Daltonics, Germany) according to the manufacturer's recommendations. The
153 bacterial test standard (BTS, Bruker) was used for instrument calibration. The cut-off scores
154 for identification were those recommended by the manufacturer.
155
156 **3. Results**

157 In all, 113 *Taylorella* species isolates and reference strains were selected based on
158 MLST data to evaluate the identification and differentiation of *T. equigenitalis* (n=85) and *T.*
159 *asinigenitalis* (n=28) by MALDI-TOF MS. The MLST results of these 113 strains yielded a
160 total of 46 sequence types (ST) distributed in 31 STs for *T. equigenitalis* (0.36 STs/strain) and
161 15 STs for *T. asinigenitalis* (0.54 STs/strain). Moreover, all strains were previously identified
162 by the gold standard culture method. Each *T. equigenitalis* strain was *T. equigenitalis*-specific
163 IIF positive, *T. equigenitalis*-specific PCR positive and *T. asinigenitalis*-specific PCR
164 negative, and conversely, each *T. asinigenitalis* strain was *T. equigenitalis*-specific IIF
165 negative, *T. equigenitalis*-specific PCR negative and *T. asinigenitalis*-specific PCR positive.
166 Among these strains, seven *T. equigenitalis* and nine *T. asinigenitalis* were selected based on

167 MLST data to generate 16 in-house *Taylorella* reference spectra to expand the existing

168 commercial Bruker database to 10 reference spectra per *Taylorella* species.

169 Colony diameter reached 1.0-1.5 mm after an incubation time of 48 h for *T.*

170 *equigenitalis* and 72 h for *T. asinigenitalis* on chocolate agar with polyvitamin supplement at

171 37±2°C in 7% (v/v) CO_2 in air. In order to carry out a MALDI-TOF MS analysis as soon as

172 the *Taylorella* species colonies were visualised, the 48 h and 72 h incubation times were

173 compared. Three isolated 48 h and 72 h colonies per strain and per sample preparation

174 procedure were processed by direct spotting and formic acid extraction, and three isolated 48

175 h and 72 h colonies per *T. asinigenitalis* strain were also processed by total protein extraction.

176 MALDI-TOF MS identifications are presented in Table 1. The MALDI-TOF MS

177 system accurately differentiated both species, *T. equigenitalis* and *T. asinigenitalis*. With the

178 existing Bruker database, the results showed typeability of 100% for *T. equigenitalis* and 93-

179 100% for *T. asinigenitalis*, and achieved accuracy of 100%. Overall, 85 (100%) *T.*

180 *equigenitalis* strains were identified in all conditions tested with a species-level identification

181 of 98.8-100%. Additionally, 26 (93%) to 28 (100%) *T. asinigenitalis* strains were identified

182 with a species-level identification of only 26-43% after formic acid extraction, 59-61% with

183 direct spotting, and 75-77% after total protein extraction. With the Bruker database expanded

184 with seven *T. equigenitalis* and nine *T. asinigenitalis* reference spectra (Bruker database,

185 expanded), all results showed 100% typeability and accuracy, and all strains per conditions

186 tested were identified with a species-level identification of 100%.

187

188 **Table 1.** *Taylorella* species identification by MALDI-TOF MS against the Bruker database or

189 Bruker database expanded with seven *T. equigenitalis* and nine *T. asinigenitalis* in-house

190 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

191 [a] Three sample preparation procedures were compared: A, direct spotting; B, formic acid extraction;

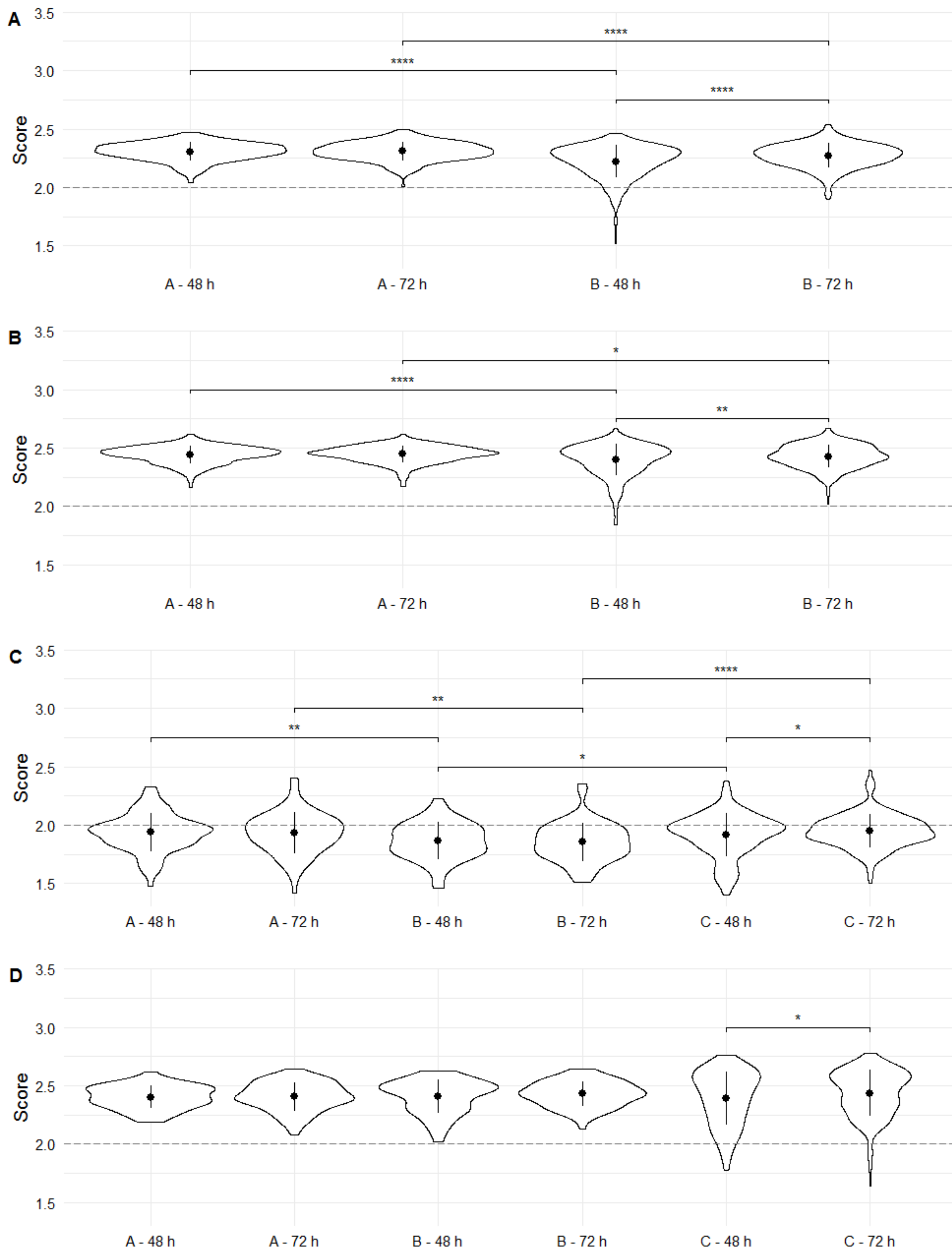
192 C, total protein extraction.

193 [b] TE, *T. equigenitalis* (n=85); TA, *T. asinigenitalis* (n=28); nt, not tested.

194 [c] MALDI-TOF MS species is not the same as the reference (scores from 1.17 to 1.51).

195 [d] MALDI-TOF MS species is the same as the reference. Manufacturer's score interpretation: <1.70, no

196 reliable identification; ≤1.99 and ≥1.70, genus-level identification; ≥2.00, species-level identification.



197 **Fig. 1.** Violin plots of MALDI-TOF MS scores between Bruker database, expanded or not,
198 bacterial incubation times, and sample preparation procedures. Spectra from 85 *T.*
199 *equigenitalis* strains **(A, B)** and spectra from 28 *T. asinigenitalis* strains **(C, D)** were analysed
200 against the Bruker database **(A, C)** or the Bruker database expanded with seven *T.*

201 *equigenitalis* and nine *T. asinigenitalis* in-house reference spectra acquired during this study
202 **(B, D)**. Three isolated 48 h and 72 h colonies per strain and per sample preparation procedure
203 were processed by direct spotting (A - 48 h and A - 72 h), formic acid extraction (B - 48 h and
204 B - 72 h), and total protein extraction (C - 48 h and C - 72 h); total protein extracts were
205 spotted in three replicates. Dots and error bars represent the mean values and standard
206 deviations, respectively. Statistically significant differences ($p < 0.05$; two-tailed Student's *t*
207 test) are marked with asterisks (*, $p < 0.05$; **, $p < 0.01$; ****, $p < 0.0001$).

208

209 MALDI-TOF MS score distributions are depicted using violin plots (Fig. 1). As
210 expected, the score values were increased for *T. equigenitalis* (Fig. 1A and 1B) and especially
211 *T. asinigenitalis* (Fig. 1C and 1D) with the expanded Bruker database (Fig. 1B and 1D)
212 compared to the Bruker database (Fig. 1A and 1C); the score values for the 85 *T. equigenitalis*
213 strains ranged from 1.52 to 2.54 (mean ± SD: 2.28 ± 0.10) with the Bruker database, and 1.84
214 to 2.67 (2.43 ± 0.09) with the expanded Bruker database, while the values for the 28 *T.*
215 *asinigenitalis* strains ranged from 1.33 to 2.47 (1.92 ± 0.16) with the Bruker database, and
216 1.64 to 2.78 (2.42 ± 0.17) with the expanded Bruker database. For *T. equigenitalis*, best
217 results were obtained when colonies were processed by direct spotting compared to formic
218 acid extraction. Significant differences across both bacterial incubation times were observed
219 only when colonies were processed by formic acid extraction, with better results for 72 h
220 colonies compared to 48 h colonies. For *T. asinigenitalis*, better results with the Bruker
221 database were obtained when colonies were processed by direct spotting and by total protein
222 extraction, compared to formic acid extraction. In contrast, no significant differences were
223 observed across the three sample preparation procedures with the expanded Bruker database.
224 However, interestingly, score distributions with total protein extraction were the most
225 extensive at values <2.00. Concerning the bacterial incubation times, significant differences

226 were observed only when colonies were processed by total protein extraction, with better
227 results for 72 h colonies compared to 48 h colonies.

228 A retrospective analysis of MALDI-TOF MS results from a veterinary diagnostic
229 laboratory (LABOCEA22) on isolated bacterial colonies of 2 330 equine samples, which were
230 all CEM-negatives by the culture method, showed 100% specificity: no *T. equigenitalis* and
231 *T. asinigenitalis* identifications were observed by MALDI-TOF MS.

232

233 **4. Discussion**

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

250 Finally, the performance of MALDI-TOF MS for the identification of *T. asinigenitalis*
251 became excellent at the species-level when the Bruker database was expanded with in-house
252 *T. asinigenitalis* reference spectra generated during this study. Importantly, the addition of the
253 *T. equigenitalis* reference spectra simply made it possible to improve the score values that
254 were already at species-level identification. Improvement of identification with an expanded
255 database has been shown in various studies on different microorganisms (Christensen et al.,
256 2012, Sogawa et al., 2012, Pérez-Sancho et al., 2018). The presence of exhaustive reference
257 MSPs in a commercial database is a critical point for this technology for microorganism
258 identification. To meet this requirement, numerous species are needed, along with expansive
259 and time-consuming work to obtain a validated database.

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

271 Two bacterial incubation times were compared during this study. Overall, the shortest
272 bacterial incubation time (48 h compared to 72 h) was sufficient to obtain excellent MALDI-
273 TOF MS performance at the species level. Essentially, differences between the bacterial
274 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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