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1 **Identification of ticks from an old collection by MALDI-TOF MS**

2

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14

15 **Abstract**

16 Objective of this study is to find the optimal conditions for preparing the samples, resulting in
17 quality, reproducible and specific MS spectra of the ticks, with a shelf life in 70% ethanol of
18 more than ten years. *Amblyomma (Am.) variegatum* species which had been stored in alcohol
19 for more than twenty years and for which numerous specimens were available were used to
20 compare the performance of four protocols tested. Spectra of insufficient quality were
21 obtained from *Am. variegatum* legs preserved in alcohol for long periods with the reference
22 protocol, named DO that we had set up years ago. The same observation was made on the
23 spectra from *Am. variegatum* legs from dry (evaporated alcohol, DO-mod protocol). With
24 new protocols named ReDO and PReDO the spectra were of good quality with high
25 intensities (> 3000 a.u.). Blind testing showed that 94%, and 93% of the spectra were
26 correctly identified with relevant log score values (LSVs ≥ 1.8), respectively for ReDO and
27 PReDO protocols. All soft ticks treated in this study by PReDO protocol exhibited low
28 intensity spectra with background noise. This study revealed that MALDI-TOF MS is able to
29 identify hard ticks stored during decades in alcohol or dry (evaporated alcohol).

30

31 **Keywords:** Tick, alcohol preservation, MALDI-TOF MS, entomology

32 **Significance of the study**

33 The correct identification of ticks, including vectors responsible for the transmission of
34 infectious diseases in humans and animals is essential for their control. MALDI-TOF MS, a
35 proteomic tool that has emerged in recent years, has become an innovative, accurate and
36 alternative tool for the identification of arthropods, including ticks. However, previous studies
37 reported that preservation of arthropods in alcohol modified the MS spectra obtained from
38 specimens of the same species freshly collected or frozenly stored. In this study, a standard
39 protocol was established for the identification of tick collections which had been stored for
40 more than ten years in alcohol. Four different protocols were assessed. The analysis of the
41 results showed that among the four protocols tested, two protocols named ReDO
42 (Rehydration and incubation of the legs in 40 μ l of HPLC water for 12 hours in a dry bath at
43 37°) and PreDO (Drying of the legs for 12 hours in a dry bath at 37°C followed by
44 rehydration and incubation in 40 μ l of HPLC water for 12 hours.) seem to be more appropriate
45 for the MALDI-TOF MS identification of ticks from old collections preserved in alcohol or
46 dry. This study is promising for the future, as it will make it possible to create a MALDI-TOF
47 MS database from a wide range of ticks which have been stored for a long time in alcohol or
48 which are dry stored in laboratories and museums around the world.

49 **1. Introduction**

50 Ticks are obligate haematophagous ectoparasites, belonging to the Arthropoda phylum, class
51 Arachnida and Order Ixodida [1]. More than 900 species have been listed, many of which are
52 parasites of wild and domestic animals. Of them, a few dozen species have acquired great
53 importance in veterinary and human medicine [2]. These ticks are vectors of several
54 pathogens, including viruses, parasites and bacteria [3–6]. In the past decade, ticks have been
55 implicated in the emergence of bacterial diseases such as borreliosis, ehrlichiosis,
56 anaplasmosis and rickettsiosis [7]. Ticks are also responsible for significant economic losses
57 in livestock production, both directly through blood spoliation from their hosts and indirectly
58 through the transmission of pathogens responsible for several diseases [8].

59 The correct identification of tick vectors is essential to optimise tick control. The
60 identification of tick species can be carried out by various methods, such as the observation of
61 morphological characteristics using dichotomous keys. This technique is reliable but has
62 several limitations, including the need for an expert in entomology and the availability of
63 appropriate identification keys. In addition to the difficulty in identifying ticks during their
64 immature stages, engorged or damaged specimens and closely related species or species
65 complexes are also factors which could hamper tick identification [9,10]. To overcome the
66 limitations of morphological observation, molecular tools have been developed targeting
67 different genes, such as the genes of the ribosomal subunit (*12S*, *16S* or *18S*), the Cytochrome
68 C oxidase Unit I (*COI*), or the internal transcribed spacer (*ITS*) [11]. However, the lack of
69 sequences of all species described in GenBank, the lack of universal primers to identify all
70 arthropods, and the cost and time involved make it difficult to use on many samples [11,12].

71 Recently, a proteomic approach using matrix-assisted laser desorption/ionisation mass
72 spectrometry (MALDI-TOF MS) has been assessed for the identification of several arthropod
73 families including *Drosophila* [13], *Culicoides* [14], mosquitoes [9], sandflies [15,16], fleas

74 [17], lice [18,19] and ticks [20,21]. This innovative approach seems promising to characterise
75 the infection status of ticks and mosquitoes [9,22,23].

76 Although MALDI-TOF MS has been considered as an efficient, rapid and robust alternative
77 tool for the identification of tick species [20,24,25], the majority of studies were conducted on
78 fresh or frozen preserved arthropods. It is interesting to note that, for specimens stored in
79 alcohol (ethanol 70%), the resulting MS spectra are modified compared to fresh or frozen
80 specimen from counter species, requiring the optimisation of the sample preparation prior to
81 the introduction of new MS spectra in the reference MS DB [25,26]. Although several
82 protocols have been established in preliminary studies for the MALDI-TOF MS identification
83 of tick species stored in alcohol, the length of time that the ticks were stored in ethanol was
84 relatively modest (≤ 3 years) [21,27]. The previously used MALDI-TOF MS protocol for
85 identification of ticks preserved in alcohol, was referred as DO protocol, in the present work.
86 It consists in removing the specimen from the alcohol and drying the tick legs overnight at
87 37°C, before homogenisation for MALDI-TOF MS analysis [27]. However, when arthropod
88 specimens have been stored for a longer period (≥ 3 years) in alcohol, a decrease in the
89 reliability of identification has been reported [21,27]. This phenomenon was mainly linked
90 with a decrease in MS peak intensity and an increase of background noise which could
91 compromised species identification [20,21,26].

92 The objective of the present study was to test the ability of MALDI-TOF MS to identify ticks
93 preserved for a long time in alcohol. In this way, firstly, existing protocols validated for
94 identification of ticks and lice stored during few years in alcohol were applied. Each time it
95 was required, an optimization of these protocols were done to improve the quality,
96 reproducibility and specificity of the MALDI-TOF MS spectra.

97

98 **2. Materials and methods**

99 *2.1. Ticks collection*

100 The different tick species came from an old collection available in our laboratory. The
101 collection of ticks began in 1956 and continued until 2005, corresponding to a collection of
102 specimens which have been stored from 15 to 64 years. Ticks were collected from a variety of
103 hosts, including domestic and wild animals or humans, and in various countries of Africa,
104 Americas, Asia and Europe. Morphological identification of the ticks was carried out down to
105 the species level by two expert entomologists. Ticks, which were morphologically classified
106 per species and collected from the same host, were preserved in the same tube with 70% (v/v)
107 alcohol. Each tube was labelled with the following information: the name of the tick species
108 and host source, the number of specimens by sex, the location and date of collection. All
109 samples were stored at room temperature. For some tubes, the alcohol evaporated over time
110 and the ticks were found in dry tubes. Details of the tick collection, origins, years and hosts
111 are summarised in Table 1.

112

113 *2.2. Tick dissection and sample preparation*

114 After rinsing each tick with distilled water and drying on sterile filter paper, the four legs
115 dissected are pooled in a single 1.5 ml micro Eppendorf tube and homogenized according to
116 the parameters of the selected protocol for MALDI-TOF MS analysis. All the ticks used in
117 this study were stored in 70% alcohol, nevertheless, the alcohol evaporated for some samples.
118 In the case that dry samples were tested, it was specified.

119

120 *2.3. Molecular validation of ticks*

121 To confirm the morphological identification of some specimens DNA from the half-idiosoma
122 of 44 tick specimens from old collections (*Am. variegatum* (50 years), *Am. hebraeum* (51
123 years), *Am. sparsum* (46 years), *Boophilus microplus* (62 years), *D. reticulatus* (15 years), *D.*

124 *variabilis* (15 years), *Hy. m. rufipes* (64 years), *Hy. impeltatum* (53 years), *I. ricinus* (16
125 years), *Rh. humeralis* (43 years), *Rh. reichenowi* (51 years) and four *Am. variegatum*
126 specimens stored since 2015 in alcohol were submitted to a standard PCR in an automated
127 DNA thermal cycler using the 16S rDNA gene by amplifying a fragment of 410 base pairs
128 [28]. Four specimens per tick species from old collections was analysed including. The
129 standard PCR and sequencing reaction contained DNA from a fresh specimen of *Am.*
130 *variegatum* laboratory-reared as a positive control.

131

132 *2.4. Optimisation of tick sample preparation prior to MALDI-TOF MS submission.*

133 The abundance of *Am. variegatum* ticks stored in alcohol or which were dry in the collection
134 led us to choose this species to test the different protocols. DO protocol (protocol 1) is the
135 protocol which has been previously established for ticks stored in 70% alcohol for up to three
136 years [27]. It consists in drying the legs of the ticks at 37°C overnight to evaporate the
137 alcohol. The dry legs are then homogenized using the TissueLyser (Qiagen, Germany), at a
138 frequency of 30 hertz during 3 x 1 minutes with glass beads (#11079110, BioSpec Products,
139 Bartlesville, OK, US) in a homogenization buffer composed of a mix (50/50) of 70% (v/v)
140 formic acid (Sigma) and 50% (v/v) acetonitrile (Fluka, Buchs, Switzerland), as previously
141 described [27]. Then the quantity of mix of the standard protocol of 40µl was reduced to 30µl,
142 25µl and 20µl while keeping the homogenization time. The second protocol tested is the DO-
143 mod Protocol (protocol 2), optimized for the MS identification of lice preserved in alcohol
144 [19]. It differs from DO protocol by decreasing the quantity of homogenization buffer (from
145 40µL to 15µL) and increasing the number of cycle homogenisation (from 3 cycles of
146 1minutes per cycle to 6 cycles). The last two protocols, named ReDO (protocol 3) and
147 PReDO (protocol 4), were developed specifically for this study. ReDO protocol was
148 developed for ticks stored in alcohol which has evaporated (dry ticks). This consists in

149 rehydrating the legs with 40µl of NaCl solution 0.9% or HPLC grade water for 12 hours. The
150 legs are then transferred to Eppendorf microtubes 1.5ml and homogenised under the same
151 conditions as DO-mod protocol. The PReDO protocol differs from the ReDO protocol by a
152 pre-drying step of the tick legs at 37°C for 12 hours to evaporate the alcohol. Then, the legs
153 were rehydrated with 40µl of HPLC-grade water for 12 hours, transferred to 1.5ml Eppendorf
154 microtube and homogenised under the same conditions as the DO-mod protocol. The quantity
155 of homogenisation buffer and time of incubation for each protocol are detailed in Table 2.
156 After the homogenisation step, a centrifugation (one minute at 2000 x g) was done and 1µL of
157 the supernatant from each sample was deposited in quadruplicate on the MALDI-TOF target
158 plate (Bruker Daltonics, Wissembourg, France). After drying, each spot was covered with
159 1µL of the CHCA matrix solution, composed of saturated α -cyano-4-hydroxycinnamic acid
160 (Sigma, Lyon, France), 50% acetonitrile (v/v), HPLC grade water and 2.5% trifluoroacetic
161 acid (Aldrich, Dorset, UK). After a few minutes drying at room temperature, the target was
162 placed in the Microflex LT MALDI-TOF mass spectrometer (Bruker Daltonics) for analysis.
163 Matrix quality (i.e., the absence of MS peaks due to matrix buffer impurities) and the
164 MALDI-TOF apparatus performance were controlled, respectively, by independently loading
165 a matrix solution in duplicate and legs from fresh *Aedes albopictus* mosquito specimens from
166 our laboratory's colonies deposited onto each MS plate as a control. The six legs from fresh
167 *Aedes albopictus* were homogenized in 30µL mix at a 3 X 1 minute homogenization cycle at
168 30 Hertz using the TissueLyser device.

169

170 2.5. MALDI-TOF MS parameters

171 Protein mass profiles were generated using a Microflex LT MALDI-TOF MS (Bruker
172 Daltonics, Germany), with a positive ion linear mode detection at a laser frequency of 50 Hz
173 over a mass range of 2 to 20 kDa. The accelerating voltage was 20 kV, and the extraction

174 delay time was 200ns [21]. Each spectrum corresponded to ions obtained from 240 laser shots
175 fired in six regions at the same point and acquired automatically using the AutoXecute
176 method of FlexControl v2.4 software (Bruker Daltonics).

177 *2.6. Analysis of spectra*

178 The spectral profiles were visualised with Flex Analysis v3.3 software and were exported to
179 ClinProTools v2.2 and MALDI-Biotyper v3.0 (Bruker Daltonics, Germany) for data
180 processing (smoothing, baseline subtraction, peak intensity values, intra-species
181 reproducibility and inter-species specificity) [26]. Each protocol was evaluated by checking
182 the quality of the spectra (values of intensity ≥ 3000 a.u. and absence of background noise),
183 intra-species reproducibility, inter-species specificity and identification rate with log score
184 values (LSVs) ≥ 1.8 . The value of the intensity (≥ 3000 a.u.) of the spectra and the absence of
185 background noise were observed using the FlexAnalysis software v.2.2. The reproducibility
186 and specificity of the spectra were assessed by unsupervised statistical tests of the MS spectra,
187 including Principal Component Analysis (PCA), cluster analysis (dendrogram) and/or
188 composite correlation index (CCI), using ClinProTools v2.2. and MALDI-Biotyper v.3.0.
189 software [26]. The identification rate with LSVs ≥ 1.8 was obtained after blind testing against
190 our database. The various statistical analyses were performed using R software, version 3.4 (R
191 Development Core Team, R Foundation for Statistical Computing, Vienna, Austria) and the
192 ggplot package.

193

194 *2.7. Creation of the reference database*

195 Based on morphological identification, between four and thirteen specimens per species and
196 per protocol were added to the homemade database of MS reference spectra (Database 1,
197 DB1) to create a second database (DB2). This DB1 contained reference spectra of *Am.*
198 *variegatum* fresh, frozen, and stored in alcohol for less than five years (Supplementary Table

199 1). Details about the arthropod species, body parts and storing conditions used to create DB 1
200 are indicated in Supplementary Table 1. The legs of each tick species with specific and
201 reproducible MS spectra were then included into a reference database of MS spectra. The
202 average spectra (Main Spectrum Profile, MSP) were created by combining the quadruplicates
203 per sample, using the automated function of the MALDI-Biotyper software (Bruker
204 Daltonics) [27]. MSPs were created based on an unbiased algorithm using peak position,
205 intensity and frequency data using the default set of parameters of the “Bio-Typer Standard
206 Method for Creating MSPs”. The reliability of species identification was estimated using the
207 log score values (LSVs) obtained from the MALDI Biotyper software v.3.0, which ranged
208 from 0 to 3. According to previous studies, LSVs greater than 1.8 were considered as being
209 reliable for species identification [21,25,26].

210

211 *2.7. Blind tests*

212 Blind tests were performed with the MS spectra of tick specimens that were treated using DO-
213 mod, ReDO, PreDO protocols. A total of 103, 134 and 456 MS spectra from tick specimens
214 treated by DO-mod, ReDO and PreDO protocols, respectively, were tested against the MS
215 reference spectra DB2. The reliability of tick species identification was estimated using the
216 log score values (LSVs) obtained from the MALDI-Biotyper software, which ranged from 0
217 to 3. A LSV was obtained for each spectrum of the samples tested. LSVs greater than 1.8
218 were considered as being reliable for species identification according to previous studies
219 [10,27].

220

221 **3. Results**

222 *3.1. Tick collection.*

223 In total, 1011 ticks sampled between 1956 and 2005 from distinct countries and 20 ticks

224 sampled in 2015 from Mali and stored in alcohol at room temperature were used. Of these,
225 931 hard ticks belonging to 17 species, and 100 soft ticks belonging to three species were
226 selected for the present study (Table 1). Interestingly, for about 13.35% of the hard tick
227 specimens (n=123), the alcohol was totally evaporated.

228 3.2. *Molecular validation of ticks*

229 A total of 49 specimens of ticks including 44 from old collections, four from 2015 and one
230 from laboratory strain, used as positive control, were randomly selected for molecular
231 analysis. PCR products were detected for 18.4% (n=9/49) of the specimens on agarose gel
232 (Supplementary figure 1), including the positive control, the fresh specimen of *Am.*
233 *variegatum* laboratory-reared. Among the 8 remaining positives 16S rDNA amplifications,
234 four corresponded to *Am. variegatum* specimens stored in alcohol since 2015, three to *D.*
235 *variabilis* stored in alcohol since 2005, and one to *Hy. m. rufipes* stored in alcohol since 1956.
236 Seven out of eight were successfully sequenced. No readable 16S rDNA sequence was
237 obtained for the *Hy. m. rufipes* PCR product.

238 BLAST analyses confirmed morphological identification of the four *Am. variegatum* (JF
239 949794, KU130402) and the three *D. variabilis* (MK742796) showing more than 99.0% of
240 sequence similarities with respective 16S rDNA sequences from GenBank (supplementary
241 table 2).

242

243 3.3. Standard protocol: *DO*

244 To evaluate the performance of the DO protocol, spectra of 20 *Am. variegatum* specimens
245 preserved for a long time (about 50 years, since 1970) in 70% alcohol homogenized in 40µl, 30µl,
246 25µl and 20µl of Mix (five specimens per volume of mix) were compared to spectra of 20 *Am.*
247 *variegatum* specimens preserved in alcohol (about 5 years, since 2015) homogenized under the
248 same conditions. The resulting MS spectra of legs of *Am. variegatum* stored for five years in

249 alcohol showed high-intensity SEP peaks (3000 a.u) and low background (fig1). Conversely,
250 long-term storage (i.e., 50 years) of ticks specimens of the same species in alcohol generated
251 low-intensity MS profiles (3000 a.u) with noise of high background regardless of the amount
252 of Mix used (Fig1). The determination of peak numbers by the ClinProTools software showed
253 a total of 21 peaks for the long-term preserved specimens. DO protocol was considered as not
254 being appropriate for the identification of ticks stored for a long time in alcohol.

255

256 3.4. DO-mod Protocol

257 To improve the quality of the spectra (intensity >3000 a.u. and low background noise), a
258 second protocol (DO-mod), used for the identification on lice preserved in alcohol [19], was
259 tested (Table 2). The combination of these two factors improved MS protein profiles by
260 limiting sample dilution in the buffer and increasing leg grind time, 47 peaks were revealed
261 by ClinProTools software. The assessment of DO-mod protocol on legs from 40 *Am.*
262 *variegatum* which had been stored for 50 years in alcohol, revealed that 85% (n=34) of the
263 MS spectra had intensity >3000 a.u. of low background (Fig 2). These 34 *Am. variegatum*
264 spectra from collections were blind tested against our reference MALDI-TOF MS database
265 (DB1). The result showed that 92.50% of the spectra were in agreement with the *Am.*
266 *variegatum* spectra in the database, but had very low LSV scores ranging from 1.12 to 1.63.
267 The DB1 was updated with spectra of 10 *Am. variegatum* specimen treated by DO-mod
268 protocol. This upgraded reference spectra database was then renamed DB2. The second blind
269 test performed with the remaining 24 specimens spectra against DB2 showed that all
270 specimens matched the correct species with LSVs ranging from 1.55 to 1.90 (Table 3). But 14
271 (58%) of the specimens had LSVs > 1.8 therefore considered reliable and 10 (42%) had
272 LSVs < 1.8 considered unreliable. DO-mod protocol was evaluated on 140 specimens of four
273 tick species including 50 *Amblyomma astrioni* (49 years old), 40 *Amblyomma tholloni* (49

274 years old), 20 *Hyalomma impeltatum* (60 years old) and 20 *Hyalomma marginatum rufipes*
275 (64 years old) stored in alcohol and ten *Am. variegatum* (24 years old) dry (i.e., the alcohol
276 has evaporated). Spectral analysis showed that 86% (112/130) of the spectra from tick species
277 stored in alcohol were good quality and reproducible (peak intensity ≥ 3000 a.u. and low
278 background) (Fig 2). However, these spectra were species-specific but not reproducible
279 within a species (Supplemental Figs. 2A and B). No good quality spectra were obtained for
280 the ten *Am. variegatum* (24 years) with evaporated (dry) alcohol. The spectra of 33 specimens
281 including 13 *Am. astrioni*, 10 *Am. tholloni*, 5 *Hy. impeltatum* and 5 *Hy. m. rufipes* were added
282 to the BD2. The blind test was performed with the remaining specimens including 32 *Am.*
283 *astrioni*, 25 *Am. tholloni*, 12 *Hy. impeltatum* and 10 *Hy. m. rufipes* against DB2. The result
284 showed that 11 (34.37%) and 22 (88%) of the spectra of *Am. astrioni* and *Am. tholloni* were
285 identified with LSVs > 1.8 (Table 3). All spectra of *Hy. impeltatum* and *Hy. m. rufipes* were
286 identified with LSVs < 1.8 . For all spectra (n=103) tested blindly for this protocol the LSVs
287 ranging from 1.29 to 2.63 (mean \pm standard deviation (SD): 1.75 ± 0.23). However, only 46%
288 (n=47) of the spectra had LSVs ≥ 1.8 (Table 3 and Supplementary Figure 2C).

289

290 3.5. ReDO Protocol

291 To improve the quality of the spectra of dry ticks, i.e., ticks stored in alcohol which has
292 evaporated over time (peak intensity < 3000 a.u. and presence of background noise) observed
293 using DO-mod protocol, ReDO protocol was developed. This protocol was first applied to
294 eight specimens of *Am. variegatum* (24 years), four of which were rehydrated with 0.9%
295 NaCl and the other four of which were rehydrated with HPLC grade water. Regardless of the
296 rehydration solvent used, the spectra obtained had high peaks (intensity ≥ 3000 a.u.) and no
297 background noise (Fig 3A). The reproducibility of the spectra was evaluated based on
298 composite correlation index (CCI) results. Higher CCI values were obtained for specimens

299 rehydrated with HPLC grade water samples (mean \pm SD: 0.66 ± 0.28) compared to those
300 rehydrated with NaCl (mean \pm SD: 0.53 ± 0.36 (Fig 3B). Therefore, HPLC grade water was
301 chosen as the rehydration solution. The result of the Statistical Analysis obtained by the
302 ClinProTools software revealed a total of 74 peaks. ReDO protocol was then assessed against
303 123 dry tick specimens, including 30 *Am. variegatum* (24 years old), 40 *Rhipicephalus*
304 *reichenowi* (51 years old), 30 *Dermacentor marginatus* (17 years old), and 23 *Rhipicephalus*
305 *longus* (50 years old). Analysis of the spectra showed that 88.7% (n=109) of them possessed
306 MS spectra with high quality (high peak intensity and no background noise) (Fig4). The
307 spectra showed intra-species reproducibility and inter-species specificity (Supplementary
308 Figs. 3A and B). Our reference database was then updated with spectra from 19 specimens
309 (Table 4). Blind testing of the spectra of the remaining 90 samples matched the correct
310 species with LSVs ranging from 1.70 to 2.54 (mean \pm SD: 2.04 ± 0.16). Interestingly, LSVs
311 higher than 1.8 were obtained for 94.44% (n=85) of the specimens, corresponding to relevant
312 identification and therefore reliable identification (Table 4 and Supplementary Fig. 3C).

313

314 3.6. PreDO Protocol

315 PreDO protocol was developed in order to have the same conservation conditions as ReDO
316 protocol, i.e. ticks stored in alcohol which has evaporated. This protocol, when applied to 30
317 *Am. variegatum* collected from the same host (stored together for 50 years in alcohol) showed
318 that 83.3% (n=25) had spectra of quality with 69 peaks as well as in intensity greater than
319 3000 a.u. of low background noise (Fig 5). Four spectra from *Am. variegatum* specimens
320 were added to our MALDI-TOF MS database. The results of the blind test of the remaining
321 21 spectra against the updated database showed that 100% matched *Am. variegatum*, with
322 LSVs ranging from 1.77 to 2.38, of which 90% (19/21) had LSVs >1.8 considered reliable for
323 correct identification .

324 PreDO protocol was then assessed against 559 specimens of twelve hard tick species and 100
325 specimens of three soft tick species (Table 5). Visualisation of the spectra using FlexAnalysis
326 software showed all spectra of soft ticks were of poor quality (intensity < 3000 a.u.,
327 background noise) (Supplementary Fig 4A). For hard ticks, 88% (491/559) specimens had
328 better quality spectra (intensity \geq 3000 au, no background noise). (Fig 5). The dendrogram
329 performed with two representative spectra of each species to assess intra-species
330 reproducibility and inter-species specificity showed the clustering of specimens of the same
331 species on the same branch (Fig 6). The MS reference database was updated with spectra
332 from 58 specimens including three to five per species (Table 5). Blind testing of the
333 remaining 429 spectra (from ticks of the same origin as the reference spectra) against the
334 updated database showed that 100% of the spectra matched with those included in the DB at
335 the correct species level, with LSVs ranging from 1.596 to 2.538 (mean \pm SD: 2.02 \pm 0.17.
336 Interestingly, 93% (n=400) of the spectra could be considered as relevantly identified with
337 LSVs higher than 1.8 (Table 5 and Supplementary Fig 4B).

338

339 **4. Discussion**

340 Over the past decade, MALDI-TOF MS, a proteomic tool, has revolutionised the field of
341 clinical microbiology and has become a gold standard for microbial identification in clinical
342 microbiology laboratories [29–31], but also in the detection pathogenic bacteria [32,33]. This
343 rapid and robust method is also used in other fields such as routine bacterial monitoring in
344 drinking water treatment plants and food quality, safety and authentication assessments in
345 food microbiology laboratories [29]. This technique is limited by the high cost of the
346 instrument (about \$150,000 but new cheaper machines are emerging) which can be offset by
347 the low cost of the analysis and the fact that the instrument is usually bought to implement
348 both microbiology and mycology platforms [34]. Although this MALDI-TOF MS technique

349 was originally designed for microbiological identification, pioneering studies over the past 15
350 years have demonstrated that it is a reliable and powerful entomological tool for identifying
351 arthropods [35]. Since then, numerous studies have demonstrated that MALDI-TOF MS is
352 remarkably robust in identifying many arthropod vectors and non-vectors, detecting
353 mislabeling of arthropods and other animals [36], improving the identification of arthropod
354 [37] and identify the origin of blood meals from engorged samples to determine the infectious
355 status of vectors [35]. Previous studies have shown that the quality of the spectra was
356 affected when arthropods were preserved in 70% alcohol [17,19,27,38]. Despite the impact of
357 alcohol on the quality of the spectra, MALDI-TOF MS makes it possible to identify these
358 arthropod specimens, provided that appropriate protocols are developed to improve the
359 quality of spectra and to create a specific database [19,21,27].

360 In regards to molecular biology, it has been repeatedly reported that the identification of
361 arthropods preserved in 70% alcohol during several years hampering nucleic acids
362 amplifications and the resort to proper protocols is require to improve DNA extraction and
363 amplification of PCR product [39,40]. Here, among the 48 tick specimens assessed for
364 molecular identification, PCR product of 16S RNA were obtained for only 8 samples
365 (16.7%), and the rate of molecular identification reached about 14.6% (n=7/48), for tick
366 specimen stored in alcohol. In the case of uniquely specimens stored more than ten years in
367 alcohol were considered (n=44), the proportion of success molecular identification dropped to
368 6.8% (n=3/44). These low results can be explained by the decrease in the amount of DNA due
369 to the prolonged storage time of the samples in 70% alcohol or the chemical and biological
370 degradation of the DNA by the alcohol [41]. But also by the fact that the DNA extraction
371 protocol used here, may not be adapted to ticks stored for a long time in alcohol. This last
372 hypothesis may be the main reason for the low percentage of DNA amplification and
373 therefore of molecular identification as observed in a previously published study [39].

374 In this study, four protocols were assessed on ticks taken from collection which had been
375 stored in alcohol for a long time (more than 10 years) as well as on dry ticks, i.e. ticks stored
376 in alcohol which had evaporated over time, in order to determine the protocol(s) that would
377 allow us to obtain quality spectra (intensity ≥ 3000 a.u., of low background noise) with intra-
378 species reproducibility and inter-species specificity. The reproducibility and specificity of the
379 species were confirmed by a Cluster Analysis showing the groupings of species by distinct
380 branches (fig6, additional figs 2A and 3A) obtained by the software MALDI-Biotyper v3.0 .
381 DO protocol (the standard protocol for ticks stored in alcohol for less than 10 years) did not
382 provide quality spectra for ticks stored long-term in alcohol (about 50 years). The impact of
383 alcohol on the quality of MS spectra could be the result of the degradation of proteins or the
384 passage of proteins from the sample to the alcohol through osmosis, thus decreasing the
385 quantity of protein in the sample.

386 In our study several modifications were made to DO protocol (the standard protocol) to
387 improve the quality of the spectra (Table 2). These modifications mainly concerned the
388 increase in homogenisation time of the samples, the decrease of the amount of grinding
389 mixture, and the addition of drying and rehydration steps. Quality spectra were obtained for
390 some tick species preserved in alcohol by varying the homogenization time and the amount of
391 grinding mixture. This improvement of the quality of the spectra had already been
392 demonstrated for lice [19] by increasing the homogenization time and decreasing the amount
393 of grinding mixture. This allowed us to extract and concentrate the proteins from the sample
394 with the DO-mod protocol. The rehydration of dry specimens allowed us to have quality
395 spectra with ReDO protocol, in contrast to DO-mod protocol where poor quality spectra
396 resulted from dry specimens. We hypothesized that this rehydration step could be at the origin
397 of the activation or reconstitution of proteins.

398 Analysis of the results showed that changing the standard protocol, i.e. modifying parameters

399 such as increasing the grinding time, decreasing the volume of grinding mixture and
400 rehydrating the samples with HPLC water, significantly improved the quality of tick leg
401 spectra. This improvement was confirmed by a statistical analysis of the peaks per protocol
402 obtained by the software FlexAnalysis v3.3, which shows a variation of the number of peaks
403 for the species of *Amblyomma variegatum* ranging from 21, 47.74 and 69 respectively per
404 protocol . These results confirm previous studies which have shown that for samples stored in
405 alcohol it is essential to develop appropriate protocols [19,27].

406 We were unable to test the three protocols on the all species of ticks because, on the one hand,
407 we did not have enough specimens for some species and, on the other, because of the lack of
408 dry specimens for all species. However, the different protocols were tested on *Am. variegatum*
409 specimens that existed in large numbers. From our results obtained for *Am. variegatum*, we
410 can say that the best protocol for the identification of dry ticks is the ReDO protocol. On the
411 other hand, for ticks preserved in alcohol the PReDO protocol seems to be the best.
412 Unfortunately, all spectra from soft ticks preserved in alcohol were of poor quality with the
413 PreDO. While the PreDO protocol allowed us to have 93% of quality spectra for hard ticks in
414 alcohol. These poor results observed in soft ticks may be due to the absence of chitin, a factor
415 favouring the rapid degradation of proteins by alcohol 70%.

416 This study shows that the identification of old ticks is possible with MALDI-TOF MS but not
417 one hundred percent because the correct identification rate i.e. with LSVs > 1.8 was 46%,
418 94% and 93% for the DO-mod, ReDO and PreDO protocols respectively. Compared to
419 molecular biology results, MALDI-TOF MS is more appropriate for the identification of old
420 collection ticks.

421 In conclusion, this study demonstrated that the MALDI-TOF MS tool can be used to identify
422 tick species from old collections which have been preserved in alcohol or when the alcohol
423 has been allowed to dry out. Our results show that ReDO protocols and PreDO appear to be

424 the most appropriate for the specimens. To fully validate these two protocols it would be
425 interesting to test them on other hard tick species from old collections and to develop other
426 protocols for soft ticks.

427

428

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432 identification of the ticks and the veterinarians. We would like to pat tribute to Guerrit
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434

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440 **Competing interests**

441 The authors declare that they have no competing interests.

442

443 **Figure legends**

444 **Figure 1:** MALDI-TOF MS spectra from the legs of *Am. variegatum* stored in alcohol for 50
445 years (A,B,C,D) and five years (E,F,G,H) and treated with the standard protocol (DO) [27]. In
446 red, representative MS spectra of *Am. variegatum* legs stored in alcohol for 50 years and
447 crushed in 40µl (A), 30µl (B), 25µl (C) and 20µl (D) solution of the homogenisation buffer.
448 In black, the spectra of *Am. variegatum* stored in alcohol for five years and crushed in 40µl
449 (E), 30µl (F), 25µl (G) and 20µl (H) of solution of the mix. a.u. = arbitrary units; m/z = mass-
450 to-charge ratio.

451
452 **Figure 2:** MALDI-TOF MS spectra from the legs of tick species treated by DO-mod
453 protocol: (A,B) *Am. variegatum* 50 years old, (C,D) *Am. astrioni* 49 years old, (E,F) *Am.*
454 *tholloni* 49 years old, (G,H) *Hy. impeltatum* 60 years old, (I,J) *Hy. m. rufipes* 64 years old,
455 and (K,L) dry *Am. variegatum* , 24 years old, are shown.

456
457 **Figure 3:** Representative MS spectra of dry *Am. variegatum*, 24 years old, according to the
458 rehydration solvent. (A): Evaluation of the reproducibility of MS spectra of 24-year-old dry
459 *Am. variegatum* legs as a function of the rehydration solution used: (red) spectra of legs
460 rehydrated with HPLC-grade water, and (green) spectra of legs rehydrated with NaCl. (B):
461 The composite correlation index (CCI) was used to compare the MS spectra of each group.
462 Four legs from specimens rehydrated with 40µl of HPLC-grade water (1-4) and four with
463 40µl of NaCl (5-8) were used. The values correspond to the correlation of the mean
464 coefficient and the respective standard deviations obtained for each group comparison.

465
466 **Figure 4:** Representative MS spectra of different ticks species treated by ReDO protocol
467 using HPLC-grade water as rehydration solvent. 24-year-old *Am. variegatum* (A,B), 50-year-

468 old *Rh. longus* (C,D), 51-year-old *Rh. reichenowi* (E,F), and 17-year-old *D. marginatus*
469 (G,H).

470 **Figure 5:** MALDI-TOF MS specific spectra of two specimens per tick species from the old
471 collection treated with PreDO protocol . Representation of the MS spectra from the legs of (1-
472 2) a 50-year-old *Am. variegatum*, (3-4) a 51-year-old *Am. hebraeum*, (5-6) a 46-year-old *Am.*
473 *sparsum*,(7-8) a 43-year-old *Am. lepidum*, (9-10) a 16-year-old *D. reticulatus*, (11-12) a 15-
474 year-old *D. variabilis*, (13-14) a 62-year-old *B. microplus*, (15-16) a 16-year-old *I. ricinus*,
475 (17-18) a 43-year-old *Hy. truncatum*, (19-20) a 53-year-old *Hy. impeltatum*, (21-22) a 61-
476 year-old *Hy.m. rufipes*, (23-24) a 50-year-old *Rh. Longus*, and (25-26) a 43-year-old *Rh.*
477 *humeralis*.

478

479 **Figure 6:** Dendrogram carried out using two MS spectra representative of 13 different tick
480 species treated with PreDO protocol from an old collection. The MSP dendrogram was
481 carried out using Biotyper v3.0 software and distance units correspond to relative similarity.

482

483 **Supplementary figure 1:** *The 1.5% agarose gel showing the migration result of the 48*
484 *specimens subjected to standard PCR. Positions 1-4 represent Am. sparsum since 1974, 5-8*
485 *Am. hebraeum since 1969, 9-12 Am. varieagtum since 1970, 13-16 Am. varieagtum since*
486 *2015, 17 -20 Hy. impeltatum since 1967, 21-24 Hy. m. rufipes since 1956, 25-28 Rh.*
487 *reichenowi since 1969 , 29-32 Rh. humeralis since 1977, 33-36 Der. variabilis since 2005,*
488 *37-40 Der. reticulatus since 2005, 41-44 Boophilus microplus since 1958 and 45-48 I. ricinus*
489 *since 2004. T-= negative control, T+= positive control (fresh Am. varieagtum), WH = size*
490 *marker and the blue line under the white bands the specimens whose DNA was amplified.*

491

492 **Supplementary figure 2:** Dendrogram carried out with four or five MS spectra representative
493 of five different tick species treated with DO-mod protocol from the old collection (A); tick
494 leg MS spectra from *Hy. m. rufipes* (yellow dots), *Hy. impeltatum* (purple dots), *Am.*
495 *variegatum* (blue dots), *Am. astrioni* (red dots), and *Am. tholloni* (green dots) treated by DO-
496 mod protocol were compared by Principal Component Analysis (C); boxplot showing the log
497 score value (LSV) distribution, the red line represents the threshold of LSV above which the
498 identification is correct and the red dot represents the mean of the LSV values (B).

499

500 **Supplementary figure 3:** Dendrogram carried out with four or five MS spectra
501 representative of five different tick species treated with ReDO protocol from the old
502 collection (A); tick leg MS spectra from *Rh. reichenowi* (yellow dots), *Rh. longus* (blue dots),
503 *Am. variegatum* (red dots), and *Der. marginatum* (green dots) treated by ReDO protocol were
504 compared by Principal Component Analysis (C); boxplot showing the log score value (LSV)
505 distribution, the red line represents the threshold of LSV above which the identification is
506 correct and the red dot represent the mean of the LSV Values (B).

507

508 **Supplementary figure 4:** MS spectra of soft ticks: 61-year-old *Argas persicus* of (a,b,c), 54-
509 year-old *Ornithodoros savignyi* of (d,e,f), 61-year-old *Otobius megnini* (g,h,i) (A). Boxplot
510 showing the log score value (LSV) distribution of hard tick species treated by PreDO protocol
511 , the red line represents the threshold of LSV above which the identification is correct and the
512 red dot represents the mean of the LSVs values (B).

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520 **Table 1:** Details about the ticks in the collection included in the present study.

Tick Genus	Tick species	Numbers of specimens tested	Date of collect (storage time in years)	Origin	Hosts
		20	1970 ¹ (50)	RCA	Buffalo
		20	2015 ¹ (5)	Mali	Cattle
		40	1970 ² (50)	RCA	Cattle
	<i>Am. variegatum</i>	10	1996 ² (24)	Guadeloupe	Cattle
		30	1970 ⁴ (50)	RCA	Cattle
		30	1996 ³ (24)	Guadeloupe	Cattle
<i>Amblyomma</i>	<i>Am. astrioni</i>	50	1971 ² (49)	RCA	Buffalo
	<i>Am. tholloni</i>	40	1971 ² (49)	RCA	Elephant
	<i>Am. sparsum</i>	40	1974 ⁴ (46)	Kenya	Breeding
	<i>Am. hebraeum</i>	35	1969 ⁴ (51)	Angleterre	Breeding
	<i>Am. lepidum</i>	20	1977 ⁴ (43)	Somalie	Cattle
	<i>D. marginatus</i>	30	2003 ³ (17)	France	Boar
<i>Dermacentor</i>	<i>D. reticulatus</i>	46	2005 ⁴ (15)	France	Not specify
	<i>D. variabilis</i>	60	2005 ⁴ (15)	Canada	Not specify
		20	1956 ² (64)	Sudan Darfour	Cattle
	<i>Hy. m. rufipes</i>	26	1959 ⁴ (61)		
<i>Hyalomma</i>	<i>Hy. truncatum</i>	45	1977 ⁴ (43)	Somalie	Cattle
	<i>Hy. impeltatum</i>	20	1960 ² (60)	Soudan blue	Cattle
	<i>Hy. impeltatum</i>	60	1967 ⁴ (53)	Tchad	Cattle
<i>Ixodes</i>	<i>I. ricinus</i>	60	2004 ⁴ (16)	Italie	Flag
<i>Boophilus</i>	<i>B. microplus</i>	60	1958 ⁴ (62)	Bresil	Cattle
<i>Rhipicephalus</i>	<i>Rh. humeralis</i>	60	1977 ⁴ (43)	Somalie	Cattle
	<i>Rh. reichenowi</i>	40	1969 ³ (51)	RCA	Warthog
		23	1970 ³ (50)	RCA	Cattle
	<i>Rh. longus</i>	46	1970 ⁴ (50)		
<i>Argas</i>	<i>Argas persicus</i>	40	1959 ⁴ (61)	Soudan Darfour	Chicken nest
<i>Ornithodoros</i>	<i>Ornithodoros megnini</i>	40	1966 ⁴ (54)	Niger	Camels
<i>Otobius</i>	<i>Otobius savignyi</i>	20	1959 ⁴ (61)	Madagascar	

Total**1031**521 ¹ Species treated by DO protocol522 ² Species treated by DO-mod protocol523 ³ Species treated by ReDO protocol524 ⁴ Species treated by PreDO protocol

525 **Table 2:** Parameters of the different protocols used in this study to treat ticks from the old
526 collection.

Protocols	Principles	Time of incubation	Length of homogenisation	Quantity of Mix	Bibliographical reference
DO (protocol 1)	Incubation at 37°C in a dry water bath	overnight	1 cycle of 3 mins at 30m/s	40µl 30µl 25µl 20µl	[27]
DO-mod (protocol 2)	Incubation at 37°C	overnight	2 cycles of 3 mins at 30 m/s	15µl 10µl	[19]
ReDO (protocol 3)	Rehydration and incubation at 37°C in 40µl NaCl or HPLC	12 hours	2 cycles of 3 mins at 30 m/s	15µl	Current work
PreDO (protocol 4)	Drying, rehydration and incubation at 37°C in 40µl HPLC	24 hours	2 cycles of 3 mins at 30 m/s	15µl	Current work

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537 **Table 3:** Tick species treated by the DO-mod protocol, number of good spectra according to species, number added to database and percentage
 538 of identification with log score values

Species (storing time in years)	Numbers tested	Excluded spectra	Numbers added to DB	Numbers used for the blind test	LSV range	Number of specimens	% of identification with LSV > 1.8	Second top species identified	LSVs of second top species [mean LSVs ± SD]
<i>Am. variegatum</i> (50)	40	06	10	24	1.815–1.904	14	58.33%	<i>Am. astrioni</i> <i>Hy. marginatum</i>	1.21±0.12
					1.558–1.782	10			
<i>Am. astrioni</i> (49)	50	05	13	32	1.801–1.934	11	34.37%	<i>Am. variegatum</i> <i>Am. gemma</i>	1.35±0.13
					1.484–1.775	21		<i>Am. sparsum</i>	
<i>Am. tholloni</i> (49)	40	05	10	25	1.889–2.637	22	88%	<i>Am. astrioni</i>	1.22±0.14
					1.394–1.759	3		<i>Am. variegatum</i>	
<i>Hy. impeltatum</i> (60)	20	03	5	12	1.194–1.637	12	00	<i>Hy. marginatum</i> <i>Am. variegatum</i>	1.36±0.13
<i>Hy. m. rufipes</i> (64)	20	05	5	10	1.291–1.587	10	00	<i>Hy. impeltatum</i> <i>Am. variegatum</i>	1.12±0.08
<i>Am. variegatum</i> (24) dry	10	10	-	-	-	-	-		
Total	180	34	43	103	1.291–2.637	103	45.63%		

539 **Table 4:** Tick species treated by the ReDO protocol, number of good spectra according to species, number added to database and percentage of
 540 identification with log score values

Species (storing time in years)	Number tested	Excluded spectra	Number added in to BD	Numbers use for the blind test	Score range	Number of specimens	% of identification with LSVs> 1.8	Second top species identified	LSVs of second top species [mean LSVs± SD]
<i>Am. variegatum</i> (24)	30	00	5	25	1.868–2.287	24	96%	<i>Am. astrioni</i>	1.39±0.12
<i>Rh. Reichenowi</i> (51)	40	06	5	29	1.80–2.385	25	86.2%	<i>Rh. sanguineus</i> <i>Rh. complanatus</i>	1.45±0.11
<i>Rh. Longus</i> (50)	23	02	5	16	1.702–1.742	04		<i>Hy. marginatum</i> <i>Rh. pusillus</i>	1.20±0.12
<i>D. marginatus</i> (17)	30	06	4	20	1.829–2.343	16	100%	<i>Hy. impeltatum</i> <i>Hy. marginatum</i>	1.41±0.11
Total	123	14	19	90	1.702–2.549	90	94.4% (85/90)		

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544 **Table 5:** Tick species treated by the PreDO protocol. number of good spectra according species. number added in data base and percentage of
 545 identification with log score values

546

Species (storing time in years)	Numbers tested	Excluded spectra	Numbers added in the BD	Numbers use blind test	Score range	% of identification with LSVs> 1.8	Second top species identified	LSVs of second top species [mean LSVs± SD]LSVs [mean LSVs± SD]
<i>Am. variegatum</i> (50)	30	5	4	21	1.802–2.383 (19)	90%	<i>Am. hebraeum</i>	1.41±0.11
					1.77–1.79 (2)		<i>Am. lepidum</i>	
<i>Am. sparsum</i> (46)	40	14	5	21	1.804–2.037 (21)	100%	<i>Am. astrioni</i> <i>Am. variegatum</i>	1.21±0.12
<i>Am. hebraeum</i> (51)	35	06	5	24	1.814–2.195 (24)	100%	<i>Am. variegatum</i>	1.16±0.12
<i>Am. lepidum</i> (43)	20	00	5	15	1.87–2.262 (12)	80%	<i>Am. hebraeum</i> <i>Am. variegatum</i>	1.42±0.12
<i>D. variabilis</i> (15)	60	15	5	40	1.71–1.763 (3)	90%	<i>Rh. annulatus</i> <i>Hy. truncatum</i> <i>Hy. impeltatum</i>	1.3±0.12
					1.803–2.284 (36)			
<i>D. reticulatus</i> (15)	46	04	5	37	1.81–2.281 (30)	81.08%	<i>D. marginatus</i> <i>Hy. marginatum</i>	1.3±0.19
<i>B. microplus</i> (62)	60	12	5	43	1.714–1.735 (7)			
<i>Rh. humeralis</i> (43)	60	3	5	52	1.812–2.538 (36)	83.7%	<i>I. ricinus</i>	1.58±0.11
					1.596–1.788 (7)			
<i>Rh. longus</i> (50)	46	00	3	43	1.862–2.281 (52)	100%	<i>D. reticulatus</i> <i>Hy. marginatum</i> <i>Rh. sanguineus</i>	1.45±0.11
					1.91–2.157 (41)		95.34%	

					1.725–1.797 (2)		<i>Rh. praetextatus</i>	1.25±0.11
<i>Ixodes. ricinus</i> (16)	60	3	5	52	1.832–2.508 (52)	100%	<i>D. reticulatus</i> <i>Rh. sanguineus</i>	1.42±0.2
<i>Hy. truncatum</i> (43)	46	2	5	39	1.817–2.345 (38)	97.5%	<i>Hy. marginatum</i>	1.52±0.14
					1.797 (1)			
<i>Hy. m. rufipes</i> (61)	26	00	5	21	1.826–2.252 (21)	100%	<i>Hy. impeltatum</i> <i>Rh. longus</i>	1.6±0.15
<i>Hy. impeltatum</i> (53)	60	13	5	42	1.80–2.203 (37) 1.714–1.785 (5)	88.09%	<i>Hy. marginatum</i> <i>Boophilus microplus</i>	1.65±0.14
<i>Argas persicus</i> (61)	40	40	-	-	-	-		
<i>Ornithodoros savignyi</i> (61)	20	20	-	-	-	-		
<i>Otobius megnini</i> (54)	40	40	-	-	-	-		
Total	689	177	62	450	1.596–2.538	93% (419/450)		

Supplementary table 1: Names of arthropod species available in our MALDI-TOF MS database. the parts used for MALDI-TOF MS analysis. the preservation methods and their infection status

Arthropods. storage methods and infection status	Compartment used for MALDI-TOF MS	Arthropod species
Ticks fresh or frozen	Leg	<i>Amblyomma variegatum</i> . <i>Rhipicephalus sanguineus</i> s.l. <i>Rhipicephalus bursa</i> . <i>Rhipicephalus pulchellus</i> . <i>Rhipicephalus sulcatus</i> . <i>Hyalomma marginatum rufipes</i> . <i>Hyalomma marginatum marginatum</i> . <i>Hyalomma impeltatum</i> . <i>Hyalomma aegyptium</i> . <i>Ixodes ricinus</i> . <i>Ixodes persulcatus</i> . <i>Dermacentor marginatus</i> . <i>Dermacentor reticulatus</i> . <i>Dermacentor silvarum</i> . <i>Haemaphysalis leachi</i> . <i>Haemaphysalis concinna</i> . <i>Haemaphysalis japonica</i> . <i>Haemaphysalis parva</i> . <i>Haemaphysalis punctata</i> . <i>Argas persicus</i> . <i>Argas lagenoplatis</i> and <i>Ornithodoros savignyi</i>
Ticks stored in alcohol	Leg	<i>Am. variegatum</i> . <i>Amblyomma gemma</i> . <i>Amblyomma cohaerens</i> . <i>Amblyomma compressum</i> . <i>Amblyomma exornatum</i> . <i>A. persicus</i> . <i>Haemaphysalis inermis</i> . <i>Hae. leachi</i> . <i>Hyalomma anatolicum anatolicum</i> . <i>Hyalomma detricum</i> . <i>Hyalomma dromedarii</i> . <i>Hyalomma excavatum</i> . <i>Hy. truncatum</i> . <i>Hy. m. rufipes</i> . <i>Hyalomma aegyptium</i> . <i>Ixodes cumulatimpunctatus</i> . <i>Ixodes hexagonus</i> . <i>I. ricinus</i> . <i>Ornithodoros sonrai</i> . <i>Rhipicephalus annulatus</i> . <i>Rhipicephalus Boophilus decoloratus</i> . <i>Rhipicephalus Boophilus microplus</i> . <i>Rhipicephalus bergeoni</i> . <i>Rh. bursa</i> . <i>Rhipicephalus complanatus</i> . <i>Rhipicephalus congolensis</i> . <i>Rhipicephalus evertsi evertsi</i> . <i>Rhipicephalus praetextatus</i> . <i>Rh. pulchellus</i> . <i>Rhipicephalus pusillus</i> and <i>Rh. sanguineus</i> s.l
Ticks infected by bacteria	Leg	<i>D. marginatus</i> infected with <i>Rickettsia slovacica</i> . <i>Rh. sanguineus</i> infected with <i>Rickettsia conorii</i> and <i>Rickettsia massiliae</i>
Mosquitoes imagos	Leg	<i>Aedes aegypti</i> . <i>Aedes albopictus</i> . <i>Aedes altermans</i> . <i>Aedes australis</i> . <i>Aedes caspui</i> . <i>Aedes cinereus</i> . <i>Aedes difouri</i> . <i>Aedes flavifrons</i> . <i>Aedes flowri</i> . <i>Aedes multiplex</i> . <i>Aedes polynesiensis</i> . <i>Aedes vexans</i> . <i>Aedes vigilax</i> . <i>Aedes excrucians</i> . <i>Anopheles arabiensis</i> . <i>Anopheles coustani</i> . <i>Anopheles claviger</i> . <i>Anopheles coluzzii</i> . <i>Anopheles funestus</i> . <i>Anopheles gambiae</i> . <i>Anopheles hyrcanus</i> . <i>Anopheles maculipennis</i> . <i>Anopheles pharoensis</i> . <i>Anopheles rufipes</i> . <i>Anopheles wellcomei</i> . <i>Anopheles ziemanni</i> . <i>Culex annulirostris</i> . <i>Culex australicus</i> . <i>Culex insigni</i> . <i>Culex modestus</i> . <i>Culex neavei</i> . <i>Culex orbostiensis</i> . <i>Culex pipiens</i> . <i>Culex quinquefasciatus</i> . <i>Culex rima</i> . <i>Culex sitiens</i> . <i>Culex watti</i> . <i>Culiseta longiareolata</i> . <i>Lutzia tigripes</i> . <i>Mansonia africana</i> . <i>Mansonia uniformis</i> . <i>Ochlerotatus excrucians</i> . <i>Ochlerotatus rusticus</i> . <i>Orthopodomyia reunionensis</i> and <i>Verrallina funereal</i>
Mosquitoes aquatic stages	Whole	<i>Ae. aegypti</i> . <i>Ae. albopictus</i> . <i>An. gambiae</i> . <i>culex hortensis</i> . <i>Cx. modestus</i> . <i>Cx. pipiens</i> . <i>Cu. longiareolata</i> and <i>Ochlerotatus caspui</i>
Lice fresh or frozeen	Cephalo-thorax	<i>Damalinia bovis</i> . <i>Damalinia caprae</i> . <i>Damalinia ovis</i> . <i>Goniodes gigas</i> . <i>Gonicotes gallinae</i> . <i>Gonoides meleagridis</i> . <i>Haematopinus eurytternus</i> . <i>Linognatus africanus</i> . <i>Linognatus vituli</i> . <i>Lipeurus caponis</i> . <i>Menacanthus stramineus</i> . <i>Menopon gallinae</i> . <i>Pediculus humanus corporis</i> and <i>Solenopotes capillatus</i>
Lice stored in alcohol	Cephalo-thorax	<i>Bovicola caprae</i> . <i>G. gallinae</i> . <i>G. gigas</i> . <i>Goniodes dissimilis</i> . <i>H. eurytternus</i> . <i>H. suis</i> . <i>Menacanthus stramineus</i> . <i>L. vituli</i> . <i>M. gallinae</i> and <i>P. humanus corporis</i>
Fleas frozeen	Cephalo-thorax	<i>Xenopsylla cheopis</i> and <i>Ctenocephalides felis</i>
Fleas stored in alcohol	Cephalo-thorax	<i>Archaeopsylla erinacei</i> . <i>C. felis</i> . <i>Ctenocephalides canis</i> . <i>X. cheopis</i> . <i>Leptopsylla taschenbergi</i> . <i>Nosopsyllus fasciatus</i> . <i>Pulex irritans</i> and <i>Stenoponia tripectinata</i>
Bedbugs fresh	Head	<i>Cimex lectularius</i> . <i>Cimex hemipterus</i> and <i>Cimex (oeciacus) hirundinis</i>

Triatominae dry	Leg	<i>Eratyrus mucronatus. Panstrongylus geniculatus. Rhodnius prolixus. Rhodnius pictipes. Rhodnius robustus and Triatoma. infestan</i>
Triatominae fresh	Leg	<i>Rhodnius robustus and Triatoma infestan</i>
Sand flies	Chephalo-thorax	<i>Phlebotomus papatasi. Phlebotomus longicuspis. Phlebotomus perfliewi. Phlebotomus perniciosus. Phlebotomus sergenti and Sergentomyia minuta</i>
Mite	Chephalo-thorax	<i>Leptotrombidium chiangraiensis. Leptotrombidium imphalum. Leptotrombidium deliense and Dermanyssus gallinae</i>
Termitidea	leg	<i>Amitermes evuncifer. Ancistrotermes cavithorax. Cubitermes orthognatus. Kallotermes flavicollis. Macrotermes belicosus. Macrotermes herus. Macrotermes ivorensis. Macrotermes subyalinus. Microcerotermes parvu. Odontotermes latericuis. Procubitermes sjostdie. Promirotermes holmegrinie. Reticulitermes lucifugus. Trinervitermes geminatus. Trinervitermes occidentalis and Trinevitermes trinervis</i>
Blattidae fresh	leg	<i>Supella longipalpa. Periplaneta americana. Blatta orientalis. Blatella germanica. and Blaptica dubia.</i>
mosquitoes blood meals	Abdomen of engorged mosquitoes	<i>An. gambiae Giles fed on Homo sapiens. Equus caballus. Ovis aries. rabbit. Balb/C. mouse. Rattus norvegicus. Canis. familiaris. Bos taurus. Capra hircus. Gallus gallus. Equus asinus. Tapirus indicus. Tapirus terrestris. Carollia perspicillata. Thraupis episcopus. Erythrocebus patas and Callithrix pygmaea blood. Ae. albopictus fed on Homo sapiens blood</i>

Supplementary table 2: Results of molecular identification of ticks specimens of old collection and *Am. variegatum* collected in 2015 using 16s rDNA gene

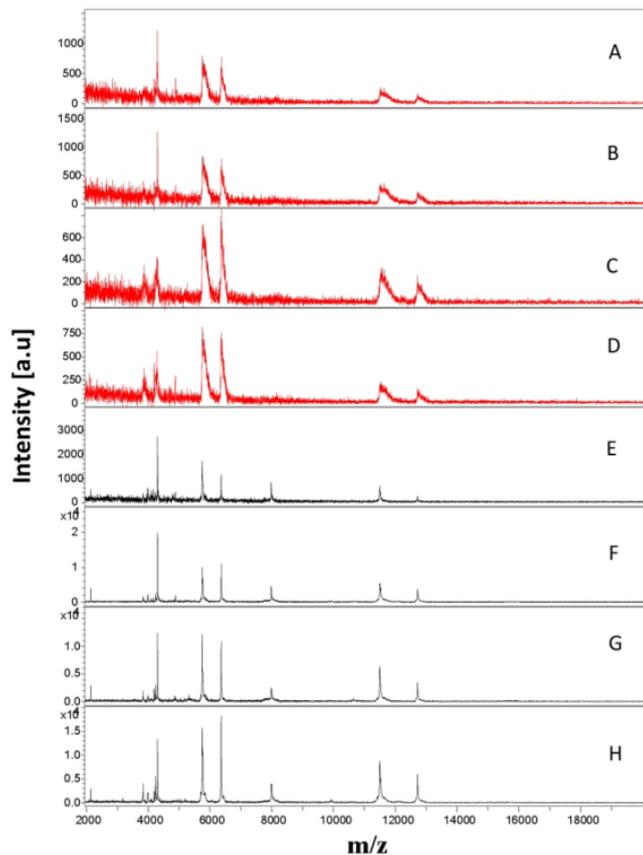
Ticks species (storing time in years)	Number tested	Number positive in standard PCR	Number of sequence obtained	coverage (%) / identity (%)	Species identified by NCBI (Accession Number)
<i>Am. variegatum</i> (6)	4	4	4	100%/99%	<i>Am. variegatum</i> (JF 949794. KU130402)
<i>Am. variegatum</i> (50)	4	0	/	/	/
<i>Am. hebraeum</i> (51)	4	0	/	/	/
<i>Am. sparsum</i> (46)	4	0	/	/	/
<i>B. microplus</i> (62)	4	0	/	/	/
<i>D. reticulatus</i> (15)	4	0	/	/	/
<i>D. variabilis</i> (15)	4	3	3	100%/99%	<i>D. variabilis</i> (MK742796)
<i>Hy. m. rufipes</i> (61)	4	1	0	/	/
<i>Hy. impeltatum</i> (60)	4	0	/	/	/
<i>I. ricinus</i> (16)	4	0	/	/	/
<i>Rh. humeralis</i> (43)	4	0	/	/	/
<i>Rh. reichenowi</i> (51)	4	0	/	/	/

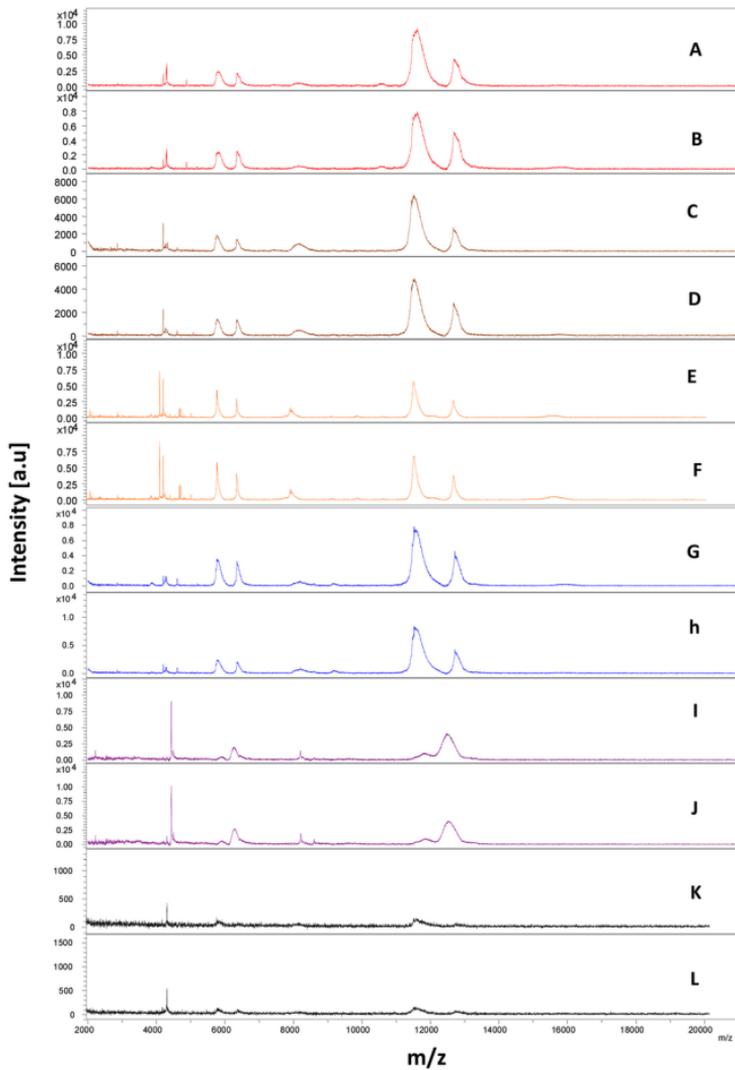
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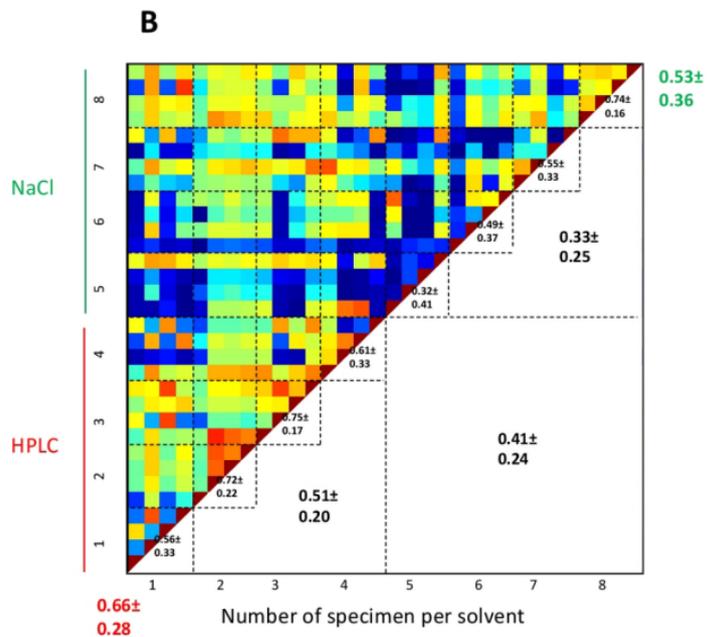
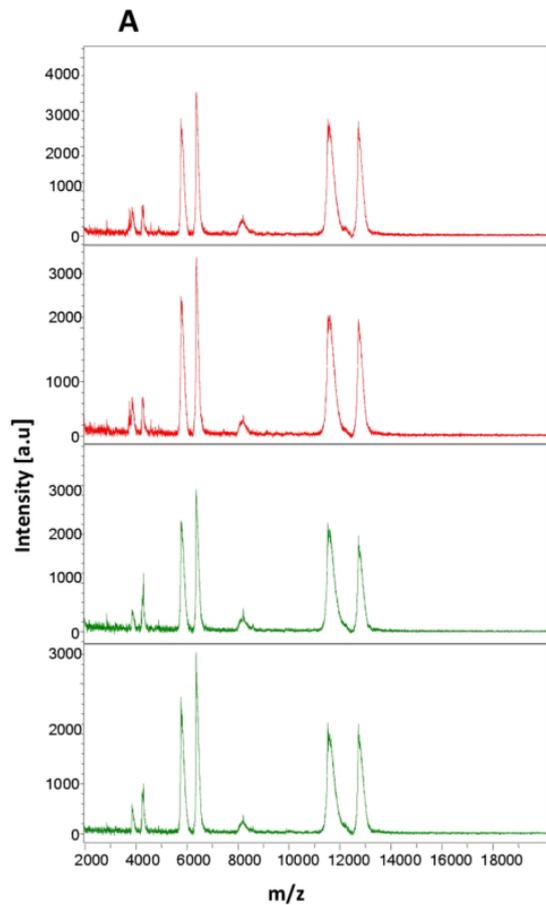
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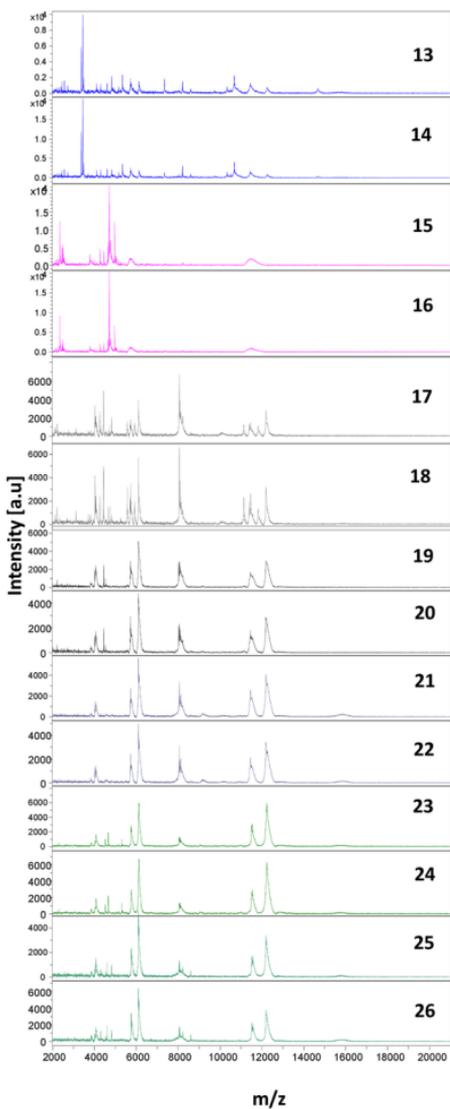
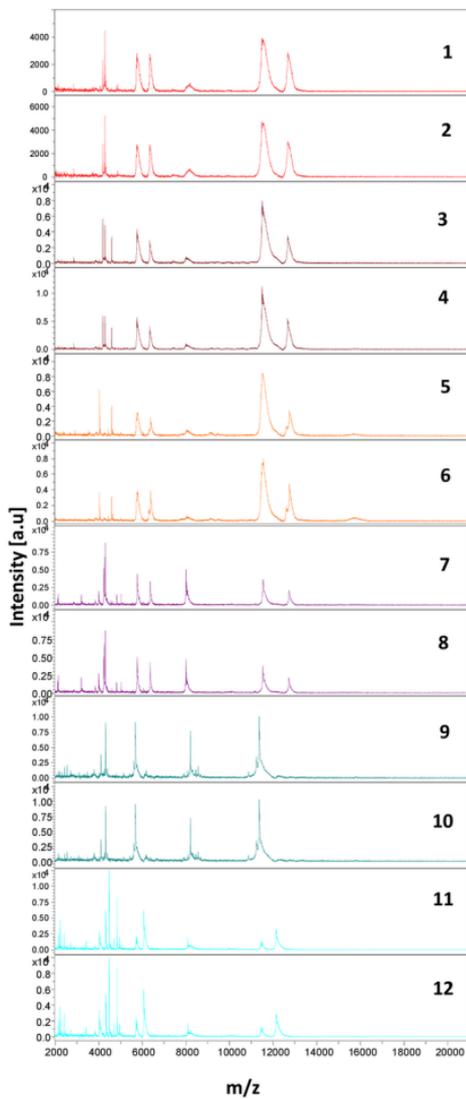
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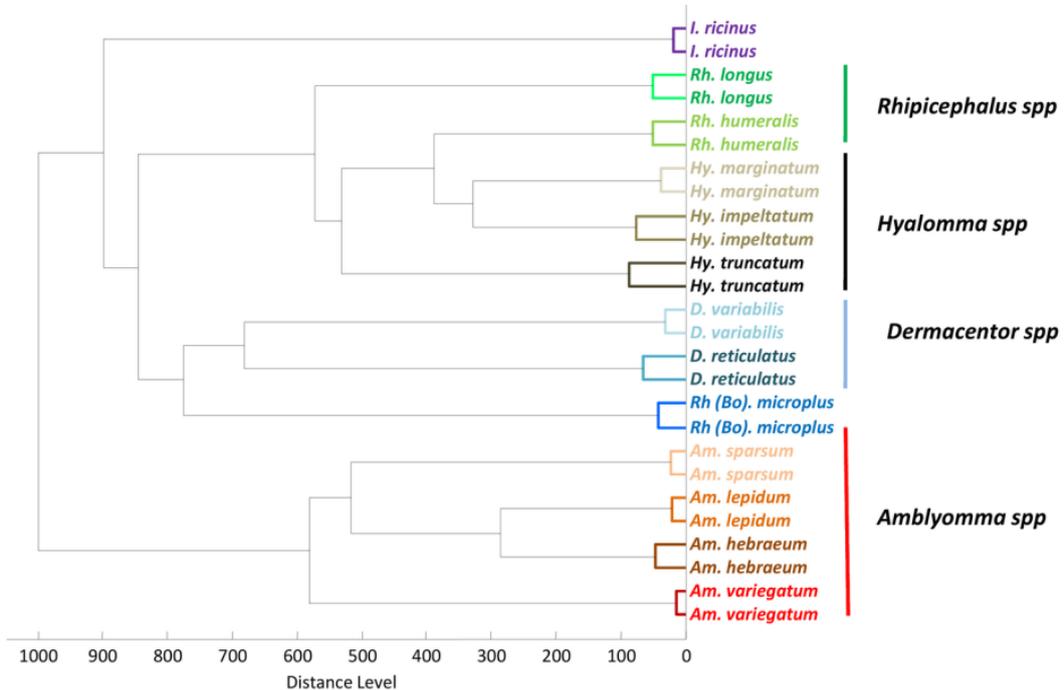








MSP Dendrogram



Assessment of preparation of ticks from an old collection for MS identification

