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1 **Detection of *Bartonella* spp. in *Cimex lectularius* by MALDI-TOF MS**

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8

9 **KEYWORDS:** *Cimex lectularius*, *Bartonella quintana*, *Bartonella henselae*, MALDI-TOF  
10 MS.

11

12 **Abstract:**

13 Bed bugs are small hematophagous insects. They are found in temperate and tropical climates  
14 around the world. Their vectorial capacity for several pathogens, including *Bartonella* spp.,  
15 has been suspected. An experimental study of artificial infection of *Cimex lectularius* with  
16 *Bartonella quintana* and *Bartonella henselae* bacteria was developed to evaluate the ability of  
17 MALDI-TOF MS to simultaneously identify bed bugs and their infectious status. This  
18 experimental study confirmed the ability of MALDI-TOF MS to identify bed bugs. In  
19 addition, it was able to differentiate between control bed bugs, bed bugs infected with  
20 *Bartonella quintana* and bed bugs infected with *Bartonella henselae*, with an identification  
21 percentage above 90%.

## 22 **Introduction**

23 Bed bugs are brown, flat parasitic [arthropods](#) belonging to the *Cimicidae* family. Males and  
24 females are strictly hematophagous and can live for 12 months without feeding [1]. The two  
25 cosmopolite species, *Cimex lectularius* and *Cimex hemipterus*, are most often found among  
26 humans [2]. Because of their hematophagous habits and intimate association with humans,  
27 bed bugs have long been [suspected](#) in disease transmission, [but their vectorial capacity has](#)  
28 [rarely been studied](#) [1].

29 Recently, [bed bugs have increased significantly in frequency and in geographical distribution](#).  
30 An increasing number of infestations have been reported in Europe and America, causing  
31 several clinical and psychological problems [1,3,4].

32 *Bartonella* spp. are fastidious, aerobic, Gram-negative bacteria. [Bartonella quintana is the](#)  
33 [agent of trench fever, known to be transmitted by the human body louse \(\*Pediculus humanus\*](#)  
34 [humanus\)](#) [5]. [Bartonella henselae is the agent of cat-scratch disease, shown to be transmitted](#)  
35 [by fleas](#) [6]. Recently, [B. quintana DNA was detected in C. hemipterus \(tropical bed bugs\) in](#)  
36 [Rwanda](#) [7]. Subsequently, [C. lectularius was shown to be a potential vector of this bacterium](#)  
37 [using an experimental study of C. lectularius bed bug infection with B. quintana](#) [5].

38 The monitoring and identification of bed bugs, as well as the detection of associated  
39 pathogens, are crucial steps for the surveillance of diseases potentially transmitted by these  
40 arthropods. Currently, bed bugs are identified primarily by observing morphological  
41 characteristics. However, the number of entomologists specialized in taxonomy is very small,  
42 and the distinction between *C. lectularius* and *C. hemipterus* cannot be made by a non-  
43 specialist [8]. [As an alternative](#), molecular tools have been used to improve identification and  
44 compensate for the limitations of morphological identification [9].

45 In recent years, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry  
46 (MALDI-TOF MS) has emerged as an efficient tool for arthropod identification [9] in

47 mosquitoes [10–12], fleas [13] and ticks [14]. More recently, MALDI-TOF MS has been  
48 successfully applied to differentiate ticks infected or not with *Borrelia crociduræ* [15] or  
49 *Rickettsia* spp. [16], fleas infected or not with *B. quintana* and *B. henselae* [17] and  
50 mosquitoes infected or not with *Plasmodium berghei* [18].

51 In this work, we set up an experimental study of bed bug infection with *B. quintana* and *B.*  
52 *henselae*. We ranked the bed bug groups according to their infectious status through  
53 molecular methods, and we evaluated the ability of MALDI-TOF to differentiate the different  
54 groups and different pathogens.

55

56

## 57 **Materials and methods**

### 58 Ethical statement

59 Human blood was obtained from the “Etablissement Français du Sang” (EFS, French Blood  
60 Establishment) accredited by the Institutional Animal Care of IHU Méditerranée Infection.

### 61 **Bed bug rearing**

62 Bed bugs (*C. lectularius*) obtained from the *Cimex* store (London, England, cimexstore.co.uk)  
63 were maintained under standard laboratory rearing conditions, as previously described [5].  
64 The breeding containers were placed in the laboratory incubator at 60% humidity and 22°C,  
65 and the same conditions were used for adults and larvae [5]. The bed bugs were fed once a  
66 week using a Hemotek artificial feeder machine (Hemotek 5W1; Discovery Workshops,  
67 Accrington, UK). The device was covered by an artificial membrane of Parafilm M (Sigma-  
68 Aldrich, Saint-Louis, Missouri, USA) and filled with 2 mL of blood [19].

### 69 ***Bartonella quintana* and *Bartonella henselae* strain**

70 All experiments with *B. quintana* and *B. henselae* were conducted inside a biosafety cabinet  
71 in a Level 2 Biosafety Room (BSL2). The two bacterial strains (strain *B. quintana* Oklahoma

72 ATCC 49793, strain *B. henselae* Marseille) were cultured on Columbia sheep blood agar  
73 plates (5%, BioMérieux, Marcy l'Etoile, France) at 37°C in an atmosphere enriched with 5%  
74 CO<sub>2</sub> [13]. After 10 days of culture, the bacteria were collected and transferred into a tube  
75 containing 400 µL of phosphate buffered saline (PBS), pH 7.2 (PBS, BioMérieux, Marcy  
76 l'Etoile, France). Two hundred microliters of each bacterial suspension mixed with two mL of  
77 human blood were used for artificial infection of bed bugs. An aliquot of two mL of human  
78 whole blood containing 200 µL of PBS was used to feed the control group [14, 17].  
79 Spectra from both strains were also obtained using MALDI-TOF MS. One colony was  
80 directly deposited on a MALDI-TOF target plate (Bruker Daltonik), and 4 spots were made  
81 for each bacterium. Each spot was covered with 2 µL of matrix solution (saturated solution of  
82 α-cyano-4-hydroxycinnamic acid in 50% acetonitrile, and 2.5% trifluoroacetic-acid) and the  
83 matrix-sample was crystallized by air-drying at room temperature for 5 minutes, as previously  
84 described (X). Measurements were performed with an Autoflex II mass spectrometer (Bruker  
85 Daltonik) equipped with a 337-nm nitrogen laser. Spectra were recorded in the positive linear  
86 mode (delay, 170 ns; ion source 1 voltage, 20 kV; ion source 2 voltage, 18.5 kV; lens voltage,  
87 7 kV; mass range, 2–20 kDa) [20].

#### 88 **Artificial infection of bed bugs**

89 Three groups were created, including a group containing 100 *C. lectularius* exposed to *B.*  
90 *quintana*, a group containing 100 *C. lectularius* exposed to *B. henselae* and a control group  
91 containing 50 *C. lectularius*.

92 Each bed bug group was fed with three successive human blood meals (every 48 hours).  
93 Control bed bugs were fed with *Bartonella*-free blood mixed with 200 µL of PBS and  
94 exposed bed bugs were fed with two mL of blood infected with 200 µL of bacterial  
95 suspension of *B. henselae* and *B. quintana* [5,17]

96 Two hundred microliters of infected blood were tested using qPCR, to ensure the presence of  
97 *B. quintana* and *B. henselae*. One hundred microliters of each inoculum were cultured on  
98 sheep blood agar to ensure bacterial viability and, at the same time, calculate the  
99 concentration by making a serial dilution of culture from  $10^{-1}$  to  $10^{10}$  [5,21].

#### 100 **DNA extraction and qPCR detection of *B. quintana* and *B. henselae* in bed bugs**

101 From the third day post infection, 10 bed bugs were collected every 48 hours in each group.  
102 Each bed bug was washed with 70% ethanol, rinsed twice with distilled water and dried with  
103 paper. Half of the abdomen was reserved for the detection of *B. quintana* and *B. henselae* by  
104 qPCR and the head was stored at  $-20^{\circ}\text{C}$  for MALDI-TOF MS tests [5,17].

105 The bed bug samples reserved for molecular biology were incubated overnight at  $56^{\circ}\text{C}$  in 180  
106  $\mu\text{L}$  of G2 buffer (Qiagen, Hilden, Germany) (30 mM Tris-Cl, 30 mM EDTA; 5% Tween 20,  
107 0.5% Triton X-100; 800 mM GuHCl) and 20  $\mu\text{L}$  of proteinase K (Qiagen, Hilden, Germany)  
108 (activity of 600 mAU/mL solution or 40 mAU/mg of protein). DNA extraction was performed  
109 in the automatic extractor EZ1 (Qiagen, Hilden, Germany). [The presence of \*B. quintana\* and](#)  
110  [\*B. henselae\* DNA in these bed bugs was assessed by \*B. quintana\* and \*B. henselae\*-specific](#)  
111 [real-time PCRs targeting the \*yopP3\* gene \[22\] and \*pap31\* gene \[23\], respectively. The](#)  
112 [presence of \*Bartonella\* spp. was tested individually in each bed bug.](#)

#### 113 **Detection of *Bartonella* spp. in *Cimex lectularius* by MALDI-TOF MS**

114 Samples reserved for MALDI-TOF MS (bed bug head) were washed a second time with  
115 HPLC-grade water and acetonitrile (Fluka, Buchs, Switzerland). They were then crushed  
116 using the automated grinding device TissueLyser (Qiagen, Hilden, Germany) 3 times for 90  
117 seconds at a frequency of 30 Hertz, with a pinch of glass powder (Sigma, Lyon, France) and a  
118 mix of 20  $\mu\text{L}$  of 70% formic acid (v/v) and 20  $\mu\text{L}$  of 50% acetonitrile (v/v) (Fluka, Buchs,  
119 Switzerland).

120 After centrifugation at 200 g for 1 minute, 1  $\mu$ L of supernatant of each sample was deposited  
121 in quadruplicate on the MALDI-TOF plate and covered by 1  $\mu$ L of CHCA matrix solution  
122 composed of saturated  $\alpha$ -cyano-4-hydroxycinnamic acid (CHCA) (Sigma, Lyon, France),  
123 50% (v/v) acetonitrile (Fluka, Buchs, Switzerland), 2.5% trifluoroacetic acid (v/v) (Aldrich,  
124 Dorset, UK) and HPLC-grade water. The target was then placed in the Microflex LT 171  
125 MALDI-TOF mass spectrometer (Bruker Daltonics) for analysis [9,17] (Laroche et al.,  
126 personal communication).

### 127 **MALDI-TOF MS parameters**

128 Protein mass profiles were generated using a Microflex LT MALDI-TOF (Bruker Daltonics,  
129 Germany) mass spectrometer, with detection in positive ion linear mode at a laser frequency  
130 of 50 Hz in a mass range 2-20 kDa. The acceleration voltage was set to 20 kV, and the time of  
131 extraction delay was 200 ns. Each spectrum corresponds to the ions obtained from 240 laser  
132 shots fired in six regions of the same deposit on the ground plate and acquired automatically  
133 using the function of the Flex Control AutoXecute V.2.4 software (Bruker Daltonics).

134 The spectra were visualized with Flex analysis v.3.3 software and exported to  
135 ClinProTools2.2 and MALDI Biotyper v.3.0 (Bruker Daltonics, Germany) for data  
136 processing. To obtain usable results, we evaluated the reproducibility of each group using the  
137 ClinProTools 2.2 software.

138 Bed bug spectra were chosen based on their reproducibility and intensity for all MS analyses,  
139 including database creation. Our database was upgraded with spectra of 4 to 11 specimens of  
140 non-infected bed bugs or bed bugs infected with *B. quintana* or *B. henselae*.

### 141 **Blind tests**

142 Blind test analysis was used to evaluate the ability of MALDI-TOF MS to distinguish the  
143 three groups. After the evaluation of the quality and the reproducibility of the spectra, a  
144 dendrogram was established for each group to evaluate the homogeneity and a high quality



145 spectra within each cluster, and high quality and reproducible MALDI-TOF MS spectra from  
146 the head of 4 *C. lectularius* controls, 5 *C. lectularius* infected with *B. henselae* and 11 *C.*  
147 *lectularius* infected with *B. quintana* were introduced into our in-house arthropod database  
148 (available on request) (Species of this database are reported in Supplementary data 1).

149 Then, a total of 34 spectra of *C. lectularius* controls, 61 spectra of *C. lectularius* infected with  
150 *B. henselae* and 62 spectra of *C. lectularius* infected with *B. quintana* were queried against  
151 the upgraded database.

## 152 **MALDI-TOF MS biomarker mass set**

153 To evaluate the reproducibility of the MS profiles, the spectra of the three groups (bed bugs  
154 infected with *B. quintana*, bed bugs infected with *B. henselae* and bed bug controls) were  
155 imported into the ClinProTools 2.2 software. For the detection of the discriminant peaks  
156 associated with infection status, we compared the average spectrum of the three categories of  
157 bed bugs. The software was used to generate a peak list for each group in the mass range of 2  
158 to 20 kDa and to identify the discriminant peaks. The parameters of the ClinProTools 2.2  
159 software for the generation of the spectra were as follows: resolution of 800; noise threshold  
160 of 2.50; maximum peak of 800 ppm and correspondence with calibration peaks of 20%. For  
161 the calculation of the peak, the peak was made on single spectra, with a signal-to-noise  
162 threshold of 2.50 and an aggregation of 800 ppm. The spectra were then analyzed with the  
163 genetic algorithm (GA) model, which provided a list of discriminant peaks. Manual  
164 inspection and peak validation by the operator gave a "recognition capability" (RC) value  
165 with the highest "cross-validation" (CV) value.

## 166 **Results**

### 167 **Acquisition of *Bartonella quintana* and *Bartonella henselae* by bed bugs**

168 The inoculum used for artificial infection with *B. quintana* contained  $3 \times 10^8$  CFU/mL  
169 bacteria and the one used for artificial infection with *B. henselae* contained  $2 \times 10^7$  CFU/mL  
170 bacteria.

171 Three days after the last infective meal, ten bed bugs from each group were subsequently  
172 collected every two days. At the end of the collections, we had a total of 80 bed bugs exposed  
173 to *B. quintana*, 80 bed bugs exposed to *B. henselae* and 40 bed bug controls.

174 Quantitative PCR results demonstrated the presence of *B. quintana* in all exposed bed bugs,  
175 with cycle threshold (Ct) values ranging from 16.19 to 27.69, and the presence of *B. henselae*  
176 in all exposed bed bugs (100% infection rate), with cycle threshold (Ct) values ranging from  
177 19.13 to 24.46. All control fleas tested negative.

#### 178 **MALDI-TOF MS for detection of *B. quintana* and *B. henselae* in bed bugs**

179 The spectra sorted by Flex Control AutoXecute V.2.4 software were analyzed by  
180 ClinProTools 2.2 software to check the reproducibility of each category (Figure 1). This  
181 analysis showed the presence of some peaks only in bed bugs infected with *B. quintana* and  
182 other peaks present only in bed bugs infected with *B. henselae* but absent in controls (Figure  
183 2).

184 The ClinProTools 2.2 software also calculated and created the principal component analysis  
185 (PCA) of the three groups of bed bugs. It compared the group of infected bed bugs with *B.*  
186 *quintana* and the control group (Figure 3.A), as well as the group of infected bed bugs with *B.*  
187 *henselae* with the control group (Figure 3.B).

188 To better identify discriminating peaks between the infected bed bugs and the control bed  
189 bugs, we used the genetic algorithm tool of the ClinProTools 2.2 software. We compared the  
190 spectra of 62 bed bugs infected with *B. quintana* and 34 spectra of control bed bugs. Similarly  
191 with the second pathogen, we compared 61 specimens of infected bed bugs with *B. henselae*  
192 and 34 control bed bugs.

193 The genetic algorithm model and operator peak picking generated 10 discriminating peaks  
194 between bed bug controls and bed bugs infected with *B. henselae* (Table 1), with a  
195 recognition capability (RC) and cross-validation (CV) of 100% for both, and nine  
196 discriminating peaks between bed bug controls and bed bugs infected with *B. quintana* (Table  
197 2), with a recognition capability (RC) of 100% and cross-validation (CV) of 98.53 % (Figure  
198 4, 5).

199 The last step of the MALDI TOF analysis was the blind test of the spectra of bed bugs against  
200 our arthropod database, previously upgraded with the reference spectra of each bed bug  
201 category (infected with *B. quintana*, infected with *B. henselae*, and controls). Bed bug species  
202 identification was correct for all reproducible spectra. Regarding the infection status,  
203 identification was correct for 56/62 *C. lectularius* infected with *B. quintana* (90.32 %), but 6  
204 of these 62 *B. quintana*-infected bed bugs were identified either as *C. lectularius* controls  
205 (n=3) or *C. lectularius* infected with *Borrelia recurrentis* (n=3).

206 The identification was correct for 55/61 *C. lectularius* infected with *B. henselae* (90.16 %),  
207 but 6 of these 61 *B. henselae*-infected bed bugs were identified either as *C. lectularius*  
208 controls (n=4) or *C. lectularius* infected with *B. quintana* (n=2). Finally, the identification  
209 was correct for 31/34 *C. lectularius* controls, but 3 of these 34 *C. lectularius* controls were  
210 identified either as *C. lectularius* infected with *B. quintana* (n=2) or *C. lectularius* infected  
211 with *B. recurrentis* (n=1) (Table 3).

212

## 213 **Discussion**

214 This work presents a preliminary study assessing the ability of MALDI-TOF MS to  
215 differentiate between non-infected bedbugs and bedbugs infected with two different species of  
216 *Bartonella* using a previously validated study of artificial infection of *C. lectularius* with  
217 *Bartonella* spp.[5]. The protocol used was developed by comparing the reproducibility and

218 quality of spectra obtained from different laboratory bed bug body parts. It was later validated  
219 on field bed bugs from very different geographical areas (urban area in south of France, rural  
220 village in Senegal) (Laroche et al., personal communication). It proved its effectiveness on  
221 infected bed bugs and controls with 88.5% of all the spectra analyzed included in the study,  
222 based on quality and reproducibility. These results are encouraging, since these spectra  
223 allowed an identification rate of bed bugs infected by *B. quintana* and *B. henselae* above 90%.  
224 In the case of slight heterogeneity within the same group, the more reference spectra one has,  
225 the more accurate the blind test analyses become. For this proof of concept step, it is  
226 necessary to keep only the best spectra to assess the performance of the tool in a blind test  
227 analysis. A validated database following a successful blind test analysis with high quality  
228 spectra means that each of these spectra can be used eventually as a reference to encompass  
229 all the future diversity encountered.

230 Two groups of bed bugs were fed blood infected with *B. henselae* or *B. quintana* separately;  
231 the qPCR showed the acquisition of both bacteria by bed bugs with a 100% infection rate, in  
232 accordance with what was previously reported [5].

233 After its success in microbiology, MALDI-TOF MS has emerged in the field of entomology  
234 and identification of arthropods. To increase the efficiency and shorten the time-to-result of  
235 MALDI-TOF MS analyses, it is necessary to standardize the sample preparation protocol,  
236 which also makes it possible to reproduce the tests in different research laboratories [8,16].  
237 This standardization encompasses several parameters; the arthropod body part used, sample  
238 washing steps and lastly, speed and frequency of grinding. These parameters vary according  
239 to the characteristics of each arthropod, requiring the development of a sample preparation  
240 protocol for MALDI-TOF MS specific to each arthropod [10,24].

241 During this work, we followed the standardized protocol implemented in our laboratory for  
242 bed bug analyses (Laroche et al., personal communication). This protocol has proven its

243 effectiveness on infected bugs and controls, with a good percentage of reproducibility  
244 (88.5%) on all spectra analyzed.

245 In this study, qPCR allowed proper classification of the three groups of bedbugs according to  
246 their infectious status. As demonstrated in previous work, bed bug heads provide reproducible  
247 spectra. Therefore, 50 specimens only were selected as controls. The database was upgraded  
248 with reference spectra of each category. The blind test showed a percentage of identification  
249 ranging from 90.16% to 91% for all three groups.

250 Regarding the identification of incorrect *Bartonella* species, we have reported in previous  
251 studies that the proteins of the infected arthropod itself could play a role in the differentiation  
252 of the two groups (infected/non-infected). Indeed, in *Plasmodium*-infected mosquitoes, a  
253 lower number of peaks and a decreased intensity of others was found compared to control  
254 mosquitoes. The role of down-regulated mosquito proteins, involved in the immune response,  
255 can be hypothesized [18]. In the mass spectrometry approach used in this study, we could not  
256 assess the exact origin of each peak. We can hypothesize that the immune response may play  
257 a role in the identification of *Bartonella* spp. in bed bugs as well. Assuming that the proteins  
258 regulated are the same for the two bacteria, we would have common peaks associated with  
259 bed bug proteins, and specific ones for each bacterium.

260 In a previous work, we evaluated the ability of MALDI-TOF MS to distinguish between fleas  
261 infected or not by *B. quintana* and *B. henselae*, and then we noticed the presence of a third  
262 group, the group of fleas exposed to the bacteria, but negative in qPCR. This category was  
263 well-differentiated from the others by MALDI-TOF MS. It had been suggested that the  
264 separation of an exposed group might be explained by the presence of the proteins of the  
265 immune response [17]. In the current study, we did not have an exposed uninfected group  
266 identified by MALDI-TOF, since the rate of infection was 100%. The few errors that have  
267 been noticed regarding the status of infection might be also linked with the immune response

268 in bed bugs, where the immune response is more important in some samples or weak or even  
269 absent in others. The listed discriminating peaks are those which allow distinction of the two  
270 groups with the lowest number of peaks. Several other peaks are actually discriminative  
271 between the two groups and are not reported. This can explain why no common  
272 discriminating peaks are found in the list presented in this study.

273 Compared to other arthropod spectra, bed bug samples demonstrate significant intra-group  
274 heterogeneity. It is therefore impossible to have a relevant identification with 2 or 3 reference  
275 spectra, as was the case for highly reproducible Australian mosquito spectra, for example  
276 [25]. An upgrade of the database with a high number of bed bug reference spectra should be a  
277 satisfactory approach to avoid misidentifications.

278 In conclusion, after a preliminary work on fleas and the present study on bed bugs, MALDI-  
279 TOF MS appears to be a promising tool for differentiating arthropods infected or not with  
280 *Bartonella*, and further complementary studies are needed to better characterize the  
281 differential proteome of infected and non-infected arthropods. Perspectives include testing  
282 this tool in the field during entomological studies. Since the MALDI-TOF MS device is  
283 bought for microbiology perspectives, its use in entomology does not require additional cost  
284 and might be very useful, given the resurgence of bed bugs.

285

286

## 287 **Tables**

| Comparison between <i>Cimex lectularius</i> infected with <i>Bartonella henselae</i> and controls |                          |                          |
|---|--------------------------|--------------------------|
| Number of peaks   | Controls Mass (m/z) (Da) | Infected Mass (m/z) (Da) |
| 1   | Absent                   | <b>4567</b>              |
| 2   | Absent                   | <b>5513</b>              |
| 3   | <b>5999</b>              | Absent                   |
| 4   | Absent                   | <b>7332</b>              |

|    |              |             |
|----|--------------|-------------|
| 5  | <b>7491</b>  | Absent      |
| 6  | <b>7742</b>  | Absent      |
| 7  | Absent       | <b>9025</b> |
| 8  | <b>10062</b> | Absent      |
| 9  | <b>11989</b> | Absent      |
| 10 | <b>14982</b> | Decreased   |

288

289 **Table 1:** Discriminant peaks between bed bug controls and bed bugs infected with *Bartonella*

290 *henselae* detected by the genetic algorithm model analyzed with ClinProTools software.

291

292

| Comparison between <i>Cimex lectularius</i> infected with <i>Bartonella quintana</i> and controls |                          |                          |
|---|--------------------------|--------------------------|
| Number of peaks   | Controls Mass (m/z) (Da) | Infected Mass (m/z) (Da) |
| 1   | Absent                   | <b>3967</b>              |
| 2   | Absent                   | <b>4574</b>              |
| 3   | Absent                   | <b>4863</b>              |
| 4   | <b>5139</b>              | Absent                   |
| 5   | <b>5997</b>              | Absent                   |
| 6   | <b>6539</b>              | Absent                   |
| 7   | Absent                   | <b>7297</b>              |
| 8   | Absent                   | <b>11132</b>             |
| 9   | Absent                   | <b>14592</b>             |

293

294 **Table 2:** Discriminant peaks between bed bug controls and bed bugs infected with *Bartonella*

295 *quintana* detected by the genetic algorithm model analyzed with ClinProTools software.

296 .

297

298

299

300

| Categories  | Total samples collected | Good quality spectra | Spectra introduced in the database | MS Good identification |
|---|-------------------------|----------------------|------------------------------------|------------------------|
| <i>Cimex lectularius</i> controls                                 | 40                      | 38                   | 4                                  | <b>31/34 (91%)</b>     |
| <i>Cimex lectularius</i> infected with <i>Bartonella henselae</i> | 80                      | 66                   | 5                                  | <b>55/61 (90, 16%)</b> |
| <i>Cimex lectularius</i> infected with <i>Bartonella quintana</i> | 80                      | 73                   | 11                                 | <b>56/62 (90, 32%)</b> |

301

302 **Table 3:** Results of the blind test analysis, after update of the MALDI-TOF MS database,  
303 analyzed by Biotyper Software.

304

305 **Figure legends:**

306 **Figure 1:** Reproducible and specific MALDI-TOF MS spectra of *Bartonella quintana* strain,  
307 *Bartonella henselae* strain, head of fresh *Cimex lectularius* infected with *Bartonella quintana*,  
308 head of fresh *Cimex lectularius* infected with *Bartonella henselae* and control bed bugs  
309 analyzed by Flex analysis 3.3 software. a.u., arbitrary units; m/z, mass-to-charge ratio.

310 **Figure 2:** The gel view tool of three categories of bed bugs. The circles frame some specific  
311 spectra of *Cimex lectularius* infected with *Bartonella quintana* (blue), *Cimex lectularius*  
312 infected with *Bartonella henselae* (green), and *Cimex lectularius* controls (red).

313 **Figure 3:** A: bed bug head MS spectra from infected *Cimex lectularius* by *Bartonella*  
314 *quintana* (green dots) and *Cimex lectularius* controls (red dots) were compared by Principal  
315 Component Analysis. B: bed bug head MS spectra from infected *Cimex lectularius* by



316 *Bartonella henselae* (green dots) and *Cimex lectularius* controls (red dots) were compared by  
317 Principal Component Analysis.

318 **Figure 4:** Comparison of MALDI-TOF MS profiles of bed bugs infected or not by *Bartonella*  
319 *quintana* using ClinProTools software. *Red* and *green* profiles correspond to average  
320 spectrum of control and infected bed bugs, respectively. *Peak masses* (Da) are indicated  
321 above the referred peak. arb.u., arbitrary units; *m/z*, mass-to-charge ratio.

322 **Figure 5:** Comparison of MALDI-TOF MS profiles of bed bugs infected or not by *Bartonella*  
323 *henselae* using ClinProTools software. *Red* and *green* profiles correspond to average  
324 spectrum of control and infected bed bugs, respectively. *Peak masses* (Da) are indicated  
325 above the referred peak. arb.u., arbitrary units; *m/z*, mass-to-charge ratio.

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### 327 **Supplementary data**

328 **Supplementary data 1:** List of all arthropod species included in our in-lab database

329

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### 336 **Competing interests:**

337 The authors declare that they have no conflicts of interest.

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