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Research paper

MALDI-TOF MS identification of *Cimex lectularius* and *Cimex hemipterus* bedbugs

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ABSTRACT

Bedbugs (*Cimex lectularius* and *C. hemipterus*) have reemerged as a major public health problem around the world. Their bites cause various skin lesions as well as discomfort and anxiety. Their role as potential vectors of various infectious agents is discussed. Accordingly, all suspected cases of bedbug infestations need to be documented thoroughly, with an unequivocal identification of the arthropods involved, if any are present. Although morphological identification is easily and quickly performed by entomologists or professionals, it can be challenging otherwise. Also, distinguishing *Cimex lectularius* and *C. hemipterus* requires entomological expertise. MALDI-TOF mass spectrometry has been recently presented as an additional tool for arthropod identification. In this study, we assess the use of MALDI-TOF MS for the identification of laboratory and wild strains of *C. lectularius* and *C. hemipterus*. Several body parts of laboratory reared *C. lectularius* specimens were used to develop a MALDI-TOF MS protocol for bedbug identification, which was later validated using five other laboratory and wild populations of *C. hemipterus* and *C. lectularius*. A total of 167*C. lectularius* and *C. hemipterus* bedbug specimens (98 laboratory specimens and 69 wild specimens) were submitted to MALDI-TOF MS analysis. 143/167 (85.63%) provided high quality MS spectra. The in-lab database was then upgraded with a total of 20 reference spectra from all bedbug populations and the rest of the MS spectra (123 bedbugs) were blind tested. All specimens were properly identified to the species level using MALDI-TOF MS and 86,25% (69/80) were aptly identified according to their origin with LSVs ranging from 1.867 to 2.861. MALDI-TOF MS appears as a reliable additional tool for the identification of these two anthropophilic species.

1. Introduction

Bedbugs are small, flattened insects belonging to the Cimicidae family and to the *Cimex* genus. All stages and both sexes are hematophagous (Cannet et al., 2015). Although its taxonomy is still being debated, the *Cimex* genus encompasses 23 currently recorded known species spread all over the world (Akhoundi et al., 2020); and according to Usinger, 1966, this genus is divided into four groups: *Cimex hemipterus* group, *C. lectularius* group, *C. pilosellus* group, and *C. pipistrelli* group. *Cimex* species usually have different geographic distribution, which is often used to identify them. *Cimex lectularius* is a cosmopolitan species but primarily live in temperate regions whereas *C. hemipterus* is mainly found in tropical areas. The *Cimex pilosellus* group occupies the Nearctic region (North America) and *C. pipistrelli* group is scattered all over the Palaearctic region. *Cimex* species are also divided into three groups according to their trophic preferences: bird bugs, bat bugs and human bugs (bedbugs). However, in the absence of their preferred host, several species can occasionally bite humans (Akhoundi et al., 2020; Kassianoff, 1937; Usinger, 1966). Both *Cimex lectularius* – the common bedbug – and *Cimex hemipterus* – the tropical bedbug – live in close association with humans, usually biting them at night (Patel and Elston, 2012). The two bedbug species can coexist in the same area of some tropical and subtropical regions such as in Florida, USA (Campbell et al., 2016), East and South Africa (Kweka et al., 2009; Myamba et al., 2002; Newberry and Mchunu, 1989; Usinger, 1966), West Africa (Aigbodion and Megbuwe, 2008; Onah Isegbe et al., 2014; Gbakima et al., 2002), Asia and the Middle East (Wang et al., 2013; Zhang et al., 2020; Rosen et al., 1987; Venkatachalam and Beldavady, 1962; Tawatsin et al., 2011) and Brazil (Nascimento, 2010; Zorrilla-Vaca et al., 2015). In NDiop and Dielmo, two villages in Senegal, *C. hemipterus* bedbugs were found in about 30% of the dwellings (Berenger and Pluot-Sigwalt, 2017). *Cimex lectularius* bedbugs were also found in this

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country, especially in Dakar (Kassianoff, 1937). In recent years, *C. hemipterus* infestations were documented in areas where they had never been described before and appears to be in Europe where *C. lectularius* is already widely established. Indeed, this tropical species has been reported in Germany (Lederer, 1950), United Kingdom (Burgess, 2003), Australia (Doggett et al., 2003), Russia (Gapon, 2016), Sweden (Persson Vinnersten, 2017), Marseille, France (Berenger and Pluot-Sigwalt, 2017) and Italy (Masini et al., 2019), sometimes even living in sympatry in the same dwelling.

The contamination of belongings as well as homes by bedbugs, has resulted in infestations of hotels, trains and other modes of transport, constantly expanding the area in which bedbugs spread (Delaunay and Pharm, 2012). Altogether, six species have been reported in Europe: *C. lectularius*, *C. hemipterus*, *C. pipistrelli*, *C. columbarius*, *C. dissimilis*, and *C. emarginatus* and three species in Africa: *C. lectularius*, *C. hemipterus*, and *C. pipistrelli* (Akhoundi et al., 2020).

To date, little evidence and even less literature is available on the potential role of bedbugs in the transmission of pathogens. More than forty microorganisms have been suspected to be transmitted by bedbugs following their detection in bedbug samples. However, these could be passively harbored in previously ingested blood without any possibility of being transmitted (Delaunay et al., 2011). Recently, two studies evaluated the competence of *C. lectularius* for the transmission of the two pathogens *Borrelia recurrentis* (El Hamzaoui et al., 2019) and *Bartonella quintana* (Leulmi et al., 2015). In laboratory settings, insects have excreted viable bacteria in their feces, highlighting the possibility of stercorarian transmission of these two bacteria. As for in vivo studies, *Trypanosoma cruzi,* the causative agent of Chagas disease, which is known to be transmitted by triatomines but which has also been detected in bedbugs, was acquired by bedbugs from parasitaemic mice and transmitted to healthy mice (Salazar et al., 2015).

Although the epidemiology of bedbug-associated microorganisms remains unclear, the presence of these insects is itself a public health issue. Pruritus and dermatitis are often associated with bedbug bites (Delaunay et al., 2009; DeShazo et al., 2012). The presence of unexplained bite marks and the experience of sleeping in a room which is suspected or confirmed to be infested often lead to psychological issues including anxiety, nervousness and insomnia (Ashcroft et al., 2015). MMoor Moreover, the eradication of a bedbug infestation may still also lead to anxiety, stress and delusory parasitosis (Simonetti et al., 2008). In such cases, as well as whenever bedbug infestations are suspected, it is important to be able to identify the insects unequivocally. Due to their small size, they can easily be misidentified as cockroaches or may not even be identified as insects at all in their early stages. Therefore, an entomologist or a professional is often needed to confirm the identification. Morphologically distinguishing bedbugs from other arthropods is not particularly challenging for people with entomological training, however, the number of trained entomologist is decreasing, and few people are able to identify bedbug species (Laroche et al., 2017b). Moreover, the differentiation between the two bedbug species (*C. lectularius* and *C. hemipterus*) is tedious (Péricart, 1973). Other relevant methods for identifying arthropods include molecular biology, although the running cost of the facilities and the comprehensiveness of reference sequence databases can be a major drawback (Lo et al., 2015).

Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) is used for the identification of biomolecules including proteins (Lin and Cai, 2018) based on the measurement of their time-of-flight after being ionized by a laser and accelerated, according to their mass-to-charge ratio, in a flight tube. Thereby, the generated spectra represents a specific fingerprint signature of the tested sample (Yssouf et al., 2016). This tool which is now widely used in routine clinical microbiology, has recently been applied to the identification of arthropods (Laroche et al., 2017b; Yssouf et al., 2016). Based on the comparison with reference spectra, it allows a low-cost, rapid identification of arthropod samples which have reference species counterparts in the database, without any specific entomological knowledge. This technology has already been successfully used to identify several arthropod families such as mosquitoes (Lawrence et al., 2018; Mewara et al., 2018; Raharimalala et al., 2017; Schaffner et al., 2014; Yssouf et al., 2013b), ticks (Boucheikhchoukh et al., 2018; Rothen et al., 2016; Yssouf et al., 2013a), fleas (Yssouf et al., 2014; Zurita et al., 2018), tsetse flies (Hoppenheit et al., 2013), sandflies (Arfuso et al., 2019; Chavy et al., 2019; Halada et al., 2018a, 2018b; Lafri et al., 2016; Mathis et al., 2015), culicoides biting midges (Kaufmann et al., 2012; Kaufmann et al., 2011) and triatomines (Laroche et al., 2017c).

In this study, we assessed the efficiency of MALDI-TOF MS for identifying laboratory and wild strains of bedbug species, and its robustness to distinguish the two *Cimex* species *C. lectularius* and *C. hemipterus.*

2. Materials & methods

2.1. Bedbug samples

A total of six different populations of two bedbug species were used in this study. Two laboratory reared strains were used for MALDI-TOF MS protocol standardization and database creation. Then, two wild strains and two other laboratory reared strains were utilized to confirm the robustness of the designed protocol for bedbug identification.

2.1.1. Bedbug rearing

The four laboratory strains of bedbugs, *C. lectularius* (London), *C. lectularius* (Germany), *C. lectularius* (Sweden) and *C. hemipterus* (Kenya) were obtained from the Cimex Store (Chepstow, UK). *Cimex lectularius* (London) and *C. hemipterus* (Kenya) (Fig. 1) were reared in our laboratory in incubators, maintaining 60% humidity and 22 °C. The bedbugs are fed once to twice a week with human blood obtained from the "Etablissement Français du Sang" (EFS, French National blood bank) accredited by the Institutional Animal Care unit within the IHU *Méditerranée Infection*. Two milliliters of blood are placed in a Hemotek artificial feeder machine (Hemotek 5 W1, Discovery Workshops, Accrington, UK) covered by an artificial membrane of Parafilm (Sigma-Aldrich, Saint-Louis, Missouri, USA), as previously described (Leulmi et al., 2015). As for the laboratory reared strains *C. lectularius* (Germany) and (Sweden), they were recently obtained alive from Cimex Store (Chepstow, UK) where they were reared for more than 40 years and since 2015 respectively. These strains were kept alive in our laboratory until they were prepared for MALDI-TOF MS analysis.

2.1.2. Wild bedbug sampling

The two wild bedbug species used in this study were collected from two different geographical locations. The wild strain of *C. lectularius* bedbugs was collected in a heavily infested home in an old building located in the city center of Marseille, France (43°17′46.227 N – 5°22′11.829E) (Fig. 2, Supplementary Data 1 and 2). All collected specimens were transported alive to our laboratory in Marseille where they were sorted and morphologically identified (Usinger, 1966) before being immediately prepared for MALDI-TOF MS analysis. *Cimex hemipterus* bedbugs were collected alive from four houses located in four neighboring rural villages in Senegal (Fig. 2, Supplementary Data 2): N'Diop (13°41′ N - 16°22′ W), Dielmo (13°43′28.052" N - 16°24′51.814" W), Medina (13°42′ N - 16°25′ W) and Passy (13°59′N - 16°16′W). All collected specimens were sorted and morphologically identified (Usinger, 1966) before being frozen at −20 °C to be transported to the laboratory in Marseille for MALDI-TOF MS analysis.

2.2. Standardization of the preparation of laboratory strain of C. lectularius for MALDI-TOF MS

Cimex lectularius (London) laboratory strain specimens were firstly rinsed for five minutes with 70% ethanol and two successive baths of

Fig. 1. Pictures of *Cimex lectularius* (A) and *Cimex hemipterus* (B) bedbugs using Zeiss Axio Zoom V16 (Zeiss, Marly le Roi, France) stereomicroscope.

distilled water before being submitted to various types of dissection to assess the quality of several body parts using MALDI-TOF MS.

Legs (*Protocol L*) of two groups including twenty *C. lectularius* (London) specimens each were retrieved and crushed in 1.5-mL microcentrifuge tubes using < 106 µm glass beads (Sigma) and a Tissue Lyser instrument (Qiagen, Hilden, Germany) at a frequency of 30 movements per second for three cycles of one minute each, either in 20 µL of 70% formic acid and 20 µL of 50% acetonitrile for the first group (*Protocol L1*) or in 15 µL formic acid and 15 µL acetonitrile for the second group (*Protocol L2*).

The heads (*Protocol H*) and cephalothorax (*Protocol CT*) of twenty *C. lectularius* (London) were dissected, immersed for 2.5 min in distilled water, rinsed with fresh distilled water and finally immersed for two minutes in 200 µL of 70% formic acid and 200 µL of 50% acetonitrile. The heads and cephalothorax were crushed separately using < 106 µm glass beads (Sigma) and a Tissue Lyser instrument (Qiagen) at a frequency of 30 movements per second for three cycles of one minute each in 20 µL of 70% formic acid and 20 µL of 50% acetonitrile in 1.5-mL microcentrifuge tubes.

All homogenates (legs, heads, and cephalothorax) were centrifuged at 7168 RCF for 30 s, and 1 µL of each supernatant was spotted onto a steel target plate (Bruker Daltonics, Germany) in quadruplicate. One microliter of a CHCA matrix suspension composed of saturated α-cyano-4-hydroxycinnamic acid (Sigma), 50% acetonitrile, 10% trifluoroacetic acid, and high-performance liquid chromatography (HPLC)-grade water was directly spotted onto each sample on the target plate to enable co-crystallization. The target plate was dried for several minutes at room temperature before being inserted into the MALDI-TOF MS instrument, as previously described (Yssouf et al., 2016).

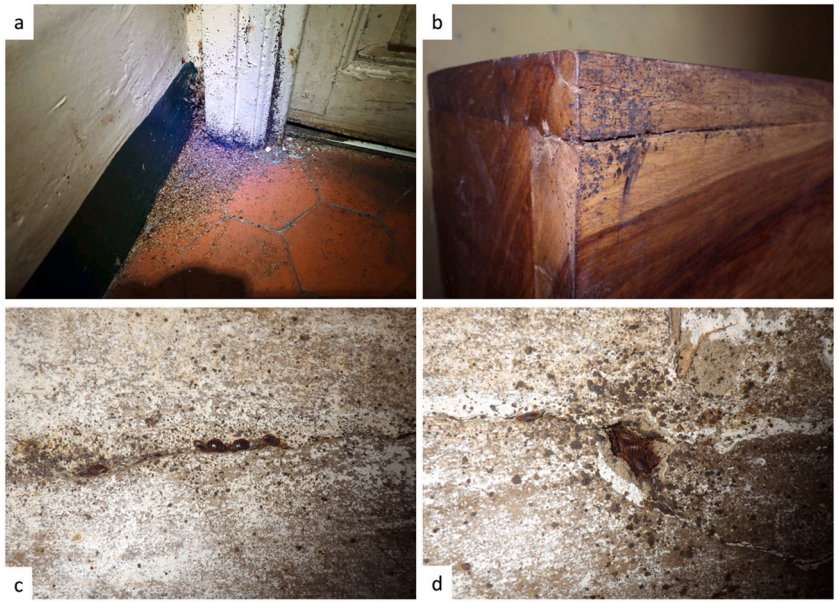

Fig. 2. Sampling of *Cimex lectularius* and *Cimex hemipterus* bedbugs in Marseille (a) and Senegal (b, c, d).

Table 1
Primers used in this study for molecular identification of wild bed bugs.

Targeted gene fragment	Reference	Primer sequences
18S	Weirauch and Munro, 2009	*18S*3f (5′-GTT CGA TTC CGG AGA GGG A-3′) *18S*Bi (5′-GAG TCT CGT TCG TTA TCG GA-3′)
COI	Folmer et al., 1994	LCO1490 (5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′) HC02198 (5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′)

2.3. MALDI-TOF MS application to the laboratory strain of Cimex hemipterus

The heads of 38 *C. hemipterus* (Kenya) were dissected, immersed for 2.5 min in distilled water, rinsed with fresh distilled water and immersed for two minutes in 200 µL of 70% formic acid and 200 µL of 50% acetonitrile. The heads were crushed separately using < 106 µm glass beads (Sigma) and a Tissue Lyser instrument (Qiagen) at a frequency of 30 movements per second for three cycles of one minute each in 20 µL of 70% formic acid and 20 µL of 50% acetonitrile in 1.5-mL microcentrifuge tubes. The MALDI-TOF MS target plates were prepared as mentioned above.

2.4. Application to other strains of Cimex lectularius and C. hemipterus bedbugs

In order to confirm the robustness of the designed protocol for bedbug identification and to check its ability to differentiate specimens of the same species from different geographical origins, it was applied to other strains of bedbugs. The protocol was firstly applied to wild specimens of *C. lectularius* and *C. hemipterus* from different origins and later using new specimens of the two reared strains previously used for the standardization of the MALDI TOF MS protocol (*C. lectularius* “London” and *C. hemipterus* “Kenya”), as described above. Finally, specimens of laboratory strains of *C. lectularius* “Germany” and “Sweden” were used to confirm the robustness of the protocol.

2.4.1. Bedbugs dissection and sample preparation for MALDI-TOF MS analysis

At first, a total of 46 wild specimens of *C. lectularius* collected in Marseille and 23 specimens of a wild strain of *C. hemipterus* collected in Senegal (10 from Dielmo, five from Passy, four from Ndiop and four from Medina) were rinsed for five minutes with 70% ethanol and two successive baths of distilled water before being dissected using sterile blades. The heads were used for MALDI-TOF MS analysis as previously described (*Protocol H*) and the MALDI-TOF MS target plates were prepared as described above. The rest of bedbugs bodies (thoraxes with legs and abdomens) were cut longitudinally into two equal parts. One body half was used for DNA extraction and molecular biology and the other half was stored frozen (−20 °C) as backup for eventual complementary tests. Afterwards, 20 new specimens of two other laboratory strains of *C. lectularius* including 10 specimens initialy from Sweden and 10 specimens initialy from Germany, along with 20 new specimens of the two laboratory strains previously used for the development of the MALDI-TOF MS protocol (10*C. lectularius* “London” and 10*C. hemipterus* “Kenya”), were tested following exactly the same rinsing, dissecting and MALDI-TOF MS protocols.

2.4.2. DNA extraction and molecular identification of wild bedbugs

In order to confirm the morphological identification of wild bedbugs from Marseille and Senegal, sixteen field collected specimens (eight from each location) previously rinsed and dissected were randomly selected and subjected to molecular analysis. The half body part of each bedbug (half of thorax with legs and abdomen) previously stored for molecular biology, was transferred to a 1.5 mL Eppendorf tube containing 180 µL of G2 lysis buffer and 20 µL of proteinase K (Qiagen) and incubated overnight at 56 °C for tissue lysis. DNA was then extracted using EZ1 robot (Qiagen) together with the EZ1 DNA Tissue kit (Qiagen) following manufacturer's recommendations. Nine *C. lectularius* (London) and seventeen *C. hemipterus* (Kenya) laboratory strains sourced from the Cimex Store (Chepstow, UK) and unambiguously identified, were extracted to be used as reference following the same DNA extraction protocol described above.

Standard PCRs amplifying a 951 base pair fragment of the *18S* rDNA gene (Weirauch and Munro, 2009) and a 710 base pair fragment of the mitochondrial cytochrome *c* oxidase subunit I gene (*COI*) (Folmer et al., 1994) (Table 1) were performed in an automated DNA thermocycler (Applied Biosystems, Foster City, CA, USA) with AmpliTaq Gold master mix (Applied Biosystems). The thermocycler program used for the amplification of the *18S* rDNA fragment consisted of initial heat activation step at 95 °C for 15 min followed by 39 cycles of denaturation 30s at 94 °C, annealing 30s at 48 °C and extension at 72 °C for 45 s; with a final extension at 72 °C for 7 min. As for the *COI* fragment, the thermocycler program used for the amplification consisted of initial heat activation step at 95 °C for 15 min followed by 39 cycles of denaturation 30s at 95 °C, annealing 30s at 50 °C and extension at 72 °C for one minute; with a final extension at 72 °C for 5 min. PCR mix without DNA was used as negative control. The amplification products were then subjected to electrophoresis through a 1.5% agarose gel stained with SYBR Safe™ and visualized with the ChemiDoc™ MP ultraviolet imager (Bio-Rad, Marnes-la-Coquette, France). PCR products of the positive samples were purified and sequenced as previously described (Tahir et al., 2016). The obtained *18S* rDNA and *COI* sequences were assembled and analyzed using the ChromasPro software (version 1.7.7) (Technelysium Pty. Ltd., Tewantin, Australia). Corrected sequences were then blasted against the NCBI GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to confirm species identity.

Consensus *18S* rDNA and *COI* sequences obtained for laboratory and wild strains of *C. lectularius* and *C. hemipterus* have been deposited in GenBank and all FASTA sequences for both amplified genes (*18S* rDNA and *COI* genes) are made available in Supplementary Data 3.

3. MALDI-TOF MS analysis

The performance of MALDI-TOF MS on each body part was assessed by visually inspecting the quality of all the MS spectra obtained for each protocol using ClinProTools 2.2 and Flex analysis v.3.3 software (Bruker Daltonics). For further analyses, only good quality spectra were selected, based on their reproducibility within each category, absence of noise, and their global intensity. A principal component analysis was created to compare good quality MS spectra obtained for each protocol using ClinProTools 2.2 software. Three reference spectra per protocol were selected to be entered into our in-lab database. For each experiment, a blind test analysis was conducted using a database containing all arthropod species of our in-lab database (Supplementary Data 4) and only the reference spectra of the protocol being tested. The Log Score Values (LSVs) were recorded for each protocol. The results of the database queries are presented as LSVs for each spectrum, corresponding to a matched degree of signal intensity of the mass spectra of the query and the reference spectra. LSVs range from 0 to 3. LSVs enable a good evaluation of the reproducibility between a queried spectrum and a reference spectrum, as it is the result of thorough comparison of peak

positions and intensity between those two spectra (MALDI BioTyper Help, Bruker). An LSV was obtained for each spectrum of the blindly tested samples. For each specimen, the spectrum with the highest LSV was selected for identification (Laroche et al., 2017c). The validated protocol was chosen to be applied to other laboratory and wild strains of *C. hemipterus* and *C. lectularius* bedbugs from different origins.

In order to visualize the similarity between MS profiles and the distances, and to assist with selecting reference spectra, a dendrogram of all tested species and strains was created using the dendrogram function of MALDI Biotyper, version 3.0. The dendrograms are based on the results of a Composite Correlation Index matrix (CCI). CCIs are calculated by dividing spectra into intervals and comparing these intervals across a data set. The composition of correlations of all intervals provides the CCI, which is used as a parameter for defining the distance between the spectra. A CCI match value of 1 represents complete correlation, whereas a CCI match value of 0 represents a lack of correlation (Laroche et al., 2017c).

Between two and five high-quality reference spectra of laboratory and wild strains of *C. hemipterus* (Kenya and Senegal) and *C. lectularius* (London, Germany, Sweden and Marseille) were entered into the database, and all *C. lectularius* and *C. hemipterus* were subjected to a blind test analysis (Table 2).

Using ClinProTools 2.2 software, a Principal Component Analysis (PCA) was created to visualize the distinction between the MS profiles of the two *Cimex* species. The same software was used to create two average spectra of *C. lectularius* and *C. hemipterus* and to identify the discriminating peaks between the two species.

4. Results

4.1. Standardization of the MALDI-TOF MS protocol on laboratory strain of C. lectularius (London)

In order to select the best protocol, all the MS spectra obtained were analyzed and compared. Neither *Protocol L1* (legs in 20 µL of 70% formic acid and 20 µL of 50% acetonitrile) nor *Protocol L2* (15µL of each) provided satisfying results. The quality of the spectra obtained was too poor, based on intensity, reproducibility and noise, to select these protocols for further analyses (Fig. 3A). Both *Protocol H* (heads) and *CT* (cephalothorax) provided high quality and reproducible spectra (Fig. 3A), however, a principal component analysis created using ClinProTools 2.2 software, showed a difference between these two protocols (Fig. 3B). The performance of the identification based on these two protocols was assessed by adding high quality reference spectra of each protocol separately to the database and submitting the remaining samples for a blind test analysis. For each blind test, only the reference spectra of the tested protocol were present in the database. The LSVs resulting from the blind test of *Protocol H* were, on average, higher and ranged from 1.818 to 2.415 (average: 2.26), compared to the LSVs of *Protocol CT* which ranged from 1.857 to 2.136 (average: 2.04). All specimens were correctly identified as bedbugs when queried against the in-lab database containing several arthropod families (Supplementary Data 4).

Protocol H was selected for further analyses. This protocol also distinguished between male and female *C. lectularius.* The average profile of the female *C. lectularius,* created by ClinProTools 2.2 software, included 92 peaks, compared to the average profile of the male *C. lectularius*, which was composed of 103 peaks.

4.2. MS identification of two laboratory strains of the Cimex species

Thirty-eight laboratory *C. hemipterus* (Kenya) specimens were subjected to MALDI-TOF MS analysis following the previously validated protocol (*Protocol H*).

Thirty of the thirty-eight specimens (79%) provided high quality spectra and were selected for further analyses. The Principal

Table 2
Final blind test results of laboratory and wild bed bugs spectra using the validated protocol.

Species	Geographical origin	Tested samples	Number of specimens with good quality spectra	Number of specimens used for database upgrade	Number of specimens used for blind test	Blind test: correct species identification	Blind test: correct strain identification	LSV obtained in blind test analysis
Cimex lectularius	Laboratory strain (London)	20	20/20 (100%)	4/20	16/20	16/16 (100%)*	–	1.818–2.415 (average: 2.112)
		10	8/10 (80%)	1/8	7/7	7/7 (100%)	6/7 (85.71%)	2.226–2.467 (average: 2.338)
	Laboratory strain (Germany)	10	10/10 (100%)	3/10	7/10	7/7(100%)	6/7(85.71%)	2.316–2.52 (average: 2.438)
	Wild strain (Marseille-France)	46	37/46 (80.43%)	2/37	35/37	35/35 (100%)	26/35 (74.28%)	2.135–2.492 (average: 2.388)
Cimex hemipterus	Laboratory strain (Sweden)	10	9/10 (90%)	2/9	7/9	7/7 (100%)	7/7 (100%)	2.266–2.5 (average: 2.399)
	Laboratory strain (Kenya)	38	30/38 (78.9%)	3/30	27/30	27/27 (100%)*	–	2.142–2.849 (average: 2.574)
	Wild strain	10	10/10 (100%)	2/10	8/10	8/8 (100%)	8/8 (100%)	2.445–2.861 (average: 2.643)
	(Senegal)	23	19/23 (82.6%)	3/19	16/19	16/16 (100%)	16/16 (100%)	1.867–2.565 (average: 2.289)
Total		167	143/167 (85.63%)	20	123	123/123 (100%)	69/80 (86.25%)	1.818–2.861

* Specimens used for MALDI-TOF MS protocol standardization and blind tested against the in-lab database upgraded with reference spectra only from laboratory strains of *C. lectularius* (London) and *C. hemipterus* (Kenya).

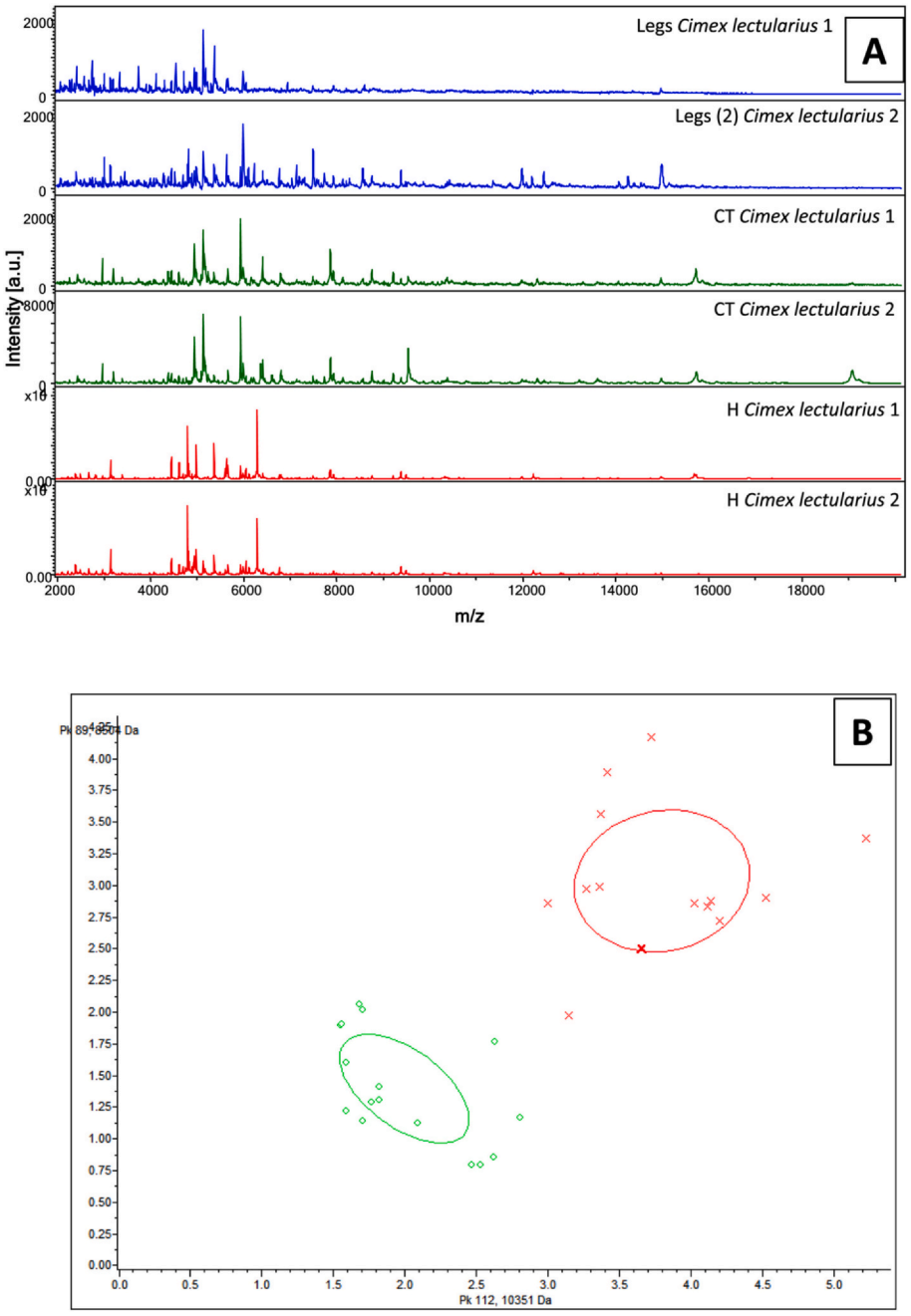

Fig. 3. (A) Representative MS profiles obtained with each protocol analyzed using the Flex analysis 3.3 software. a.u.: arbitrary units; *m/z*: mass-to-charge ratio; H: head protocol; CT: cephalothorax protocol. (B) MALDI-TOF MS distinction of spectra from Protocol H using *Cimex lectularius* bed bugs heads (crosses; 4 specimens) and Protocol CT using *Cimex lectularius* bed bugs Cephalothorax (circles; 4 specimens) illustrated on principal component analysis using ClinProTools 2.2 software. a.u.: arbitrary units; m/z: mass-to-charge ratio.

Component Analysis (PCA), including randomly selected spectra from 20*C. lectularius* (London) and 20*C. hemipterus* (Kenya) (10 male and 10 female bedbugs each), revealed a clear distinction of their MS profiles (Fig. 4A) as confirmed by the dendrogram (Fig. 5). Genetic algorithm analysis, processed with ClinProTools software, revealed that a minimum of two peaks (5908 and 5942 Da) were necessary to distinguish *C. lectularius* from *C. hemipterus,* with a "recognition capability" (RC) value of 100%, coupled with a 100% "cross-validation" (CV) value. Nevertheless, visual inspection of average spectrum of all *C. lectularius* (London) spectra compared to the average spectrum of all *C. hemipterus* (Kenya) spectra revealed many more discriminative peaks (Fig. 4B).

The average profile of the female *C. hemipterus,* created using ClinProTools 2.2 software, included 81 peaks, compared to the average profile of the male *C. hemipterus* which was composed of 103 peaks. These 103 peaks were, however, different than the 103 peaks of the male *C. lectularius* profiles, since both male species were easily separated using a PCA (Supplementary Data 5). Ten peaks were found to be discriminative between male and female *C. hemipterus* using the genetic algorithm (GA) model included the ClinProTools 2.2 software and are listed in Supplementary Data 6. Manual inspection and validation of the selected peaks by the operator gave a RC value of 100%, coupled with a 99.44% CV value. These values reflect the ability to distinguish between different spectra groups based on the chosen discriminative peaks. The same analysis was also performed in order to highlight discriminative peaks between average male and female *C. lectularius*

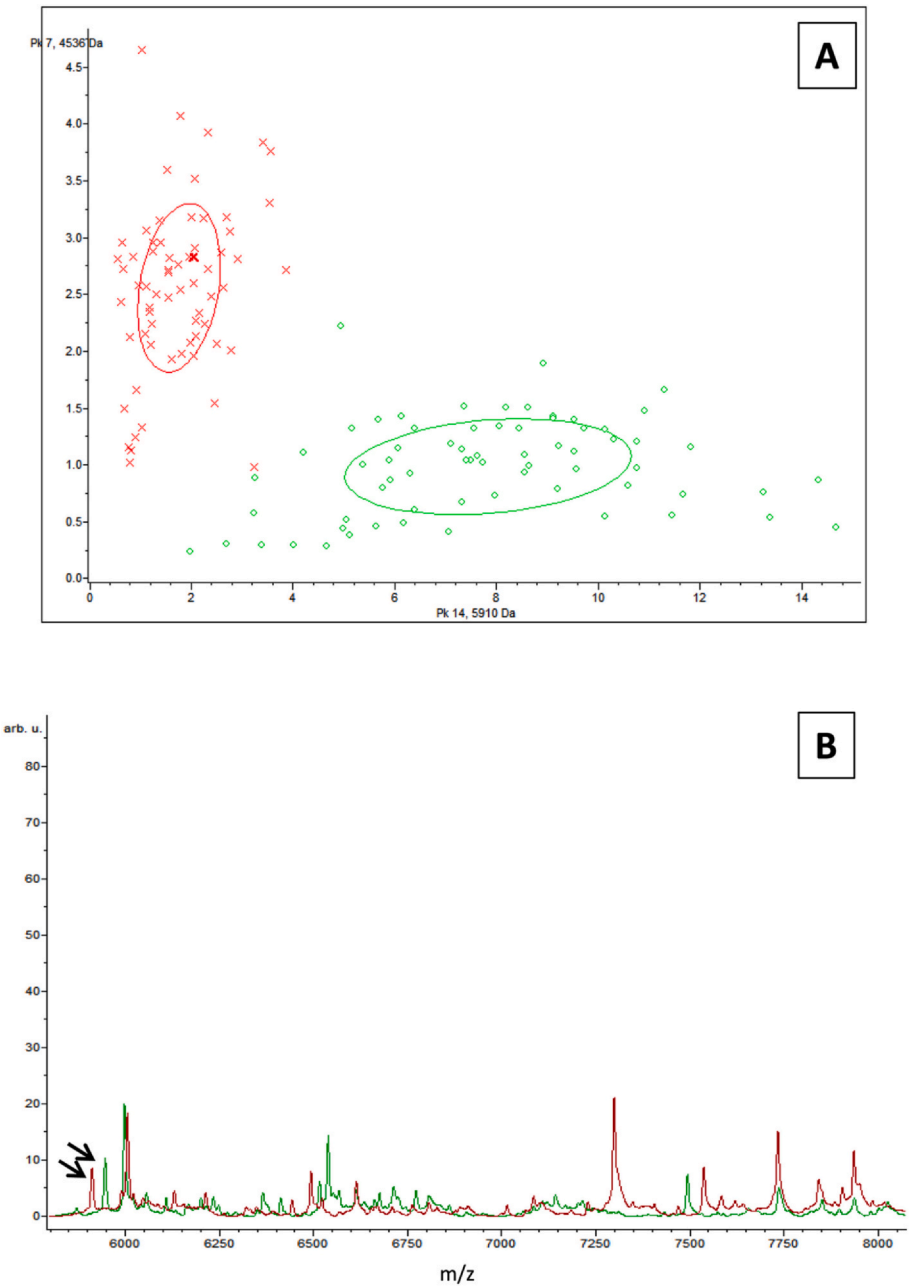

Fig. 4. (A) MALDI-TOF MS distinction of spectra from *Cimex lectularius* (crosses; 10 female, 10 male specimens) and *Cimex hemipterus* (circles; 10 female, 10 male specimens) bed bugs illustrated on principal component analysis using ClinProTools 2.2 software. a.u.: arbitrary units; m/z: mass-to-charge ratio. (B) Superimposed average spectra of *Cimex lectularius* (red) and *Cimex hemipterus* (green) with minimum discriminative peaks indicated with black arrows, obtained with ClinProTools software. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

profiles. Ten peaks were also selected, as they provided 100% RC value with 96.85% CV value. PCA analysis confirmed MS distinction between male and female bedbugs of both species (Fig. 6) and further information regarding discriminative peaks is presented in Supplementary Data 6.

4.3. MS identification of wild and laboratory strains of the Cimex species

The laboratory strains *C. lectularius* (Germany), *C. lectularius* (London), *C. lectularius* (Sweden) and *C. hemipterus* (Kenya) were obtained from the Cimex Store where they were unequivocally identified. As for the field collected bedbugs from Marseille and Senegal, they were morphologicaly identified in our laboratory by an entomologist and later using molecular biology. Morphological identification of *C. lectularius* and *C. hemipterus* is mainly based on thorax dimensions. For *C. hemipterus,* pronotum is less than 2.5 times as wide as long, contrary to *C. lectularius,* for which pronotum is more than 2.5 times as wide as long (Fig. 7) (Usinger, 1966).

Firstly, forty-six *C. lectularius* collected in Marseille, France and twenty-three *C. hemipterus* collected in Senegal were then submitted for MALDI-TOF MS analysis following the earlier validated *Protocol H*. Thirty-seven of forty-six (80%) wild *C. lectularius* (Marseille) specimens and nineteen of the twenty-three (82%) wild *C. hemipterus* (Senegal) specimens provided reproducible and high-quality spectra. In a second time, ten specimens of each laboratory strains *C. lectularius* (Germany), *C. lectularius* (Sweden), *C. lectularius* (London) and *C. hemipterus* (Kenya), have been submitted for MALDI-TOF MS analysis applying the *Protocol H*. All (100%) *C. lectularius* (Germany), nine of ten (90%) *C.*

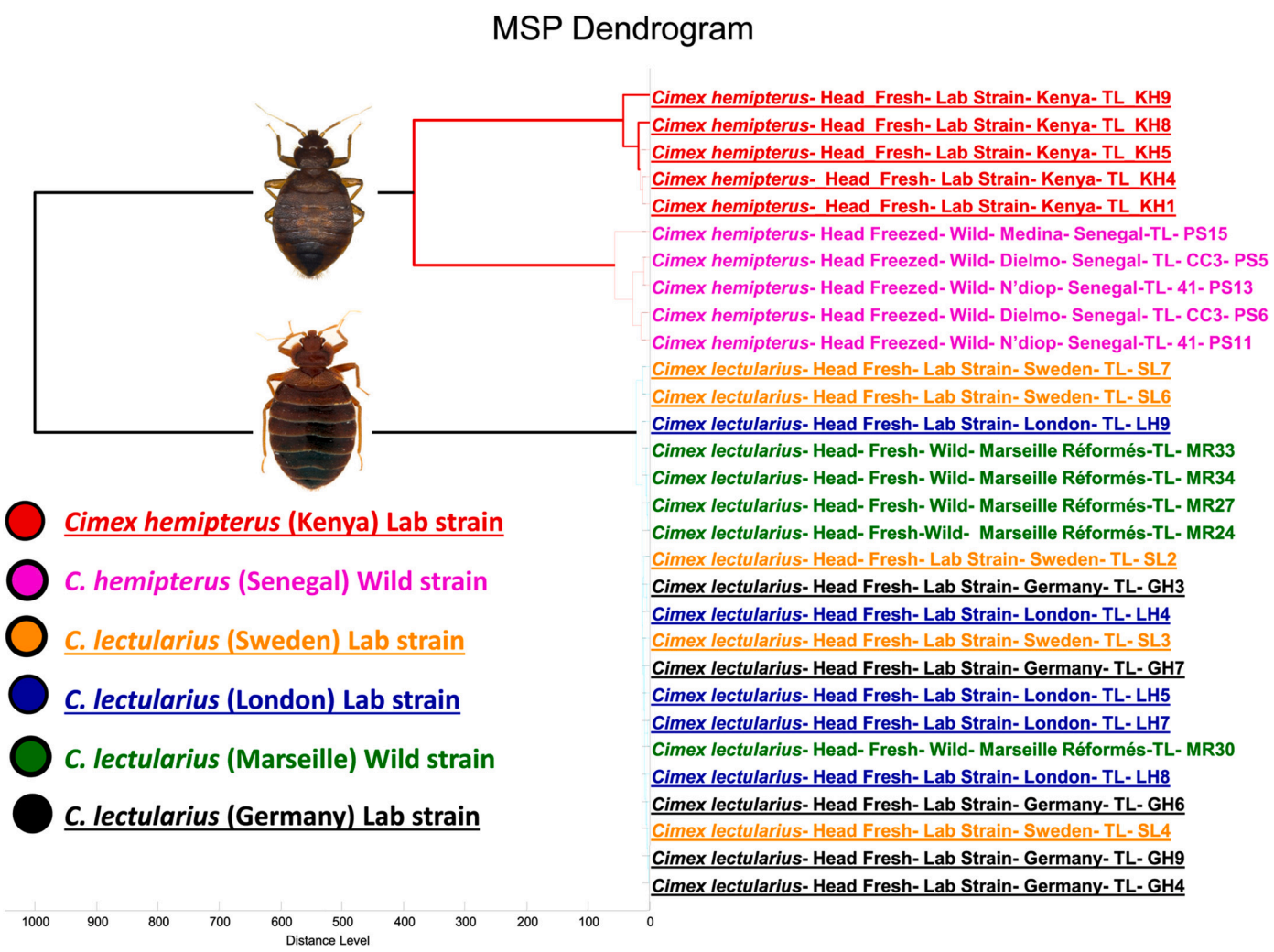

Fig. 5. Dendrogram obtained by cluster analysis of spectra obtained from 5 randomly selected *Cimex lectularius* and *Cimex hemipterus* specimens from the six populations: London laboratory strain, Germany laboratory strain, Sweden laboratory strain, Kenya laboratory strain, Marseille wild population, Senegal wild population. Laboratory strains are underlined.

lectularius (Sweden), eight of ten (80%) *C. lectularius* (London) and all (100%) *C. hemipterus* (Kenya) specimens provided reproducible and high-quality spectra.

The dendrogram which was created from randomly selected high-quality spectra from all the wild and laboratory bedbug populations of the two species revealed a specific clustering of the MS profiles according to the species. It revealed also a specific clustering of the MS profiles according to the populations for *C. hemipterus*, but such clustering was not observed for *C. lectularius* populations (Fig. 5). All good quality spectra from specimens used for protocol standardization were submitted for a blind test analysis against the in-lab database, which had been firstly upgraded with reference spectra from laboratory strains of *C. lectularius* (London) and *C. hemipterus* (Kenya) only. Spectra from specimens from different origins – used for MALDI-TOF MS protocol validation – were queried against the in-lab database which had been upgraded with reference spectra from the six populations of laboratory and wild strains of *C. lectularius* (London, Germany, Sweden and Marseille) and *C. hemipterus* (Kenya and Senegal).

First blind test queries of laboratory strains used for MALDI-TOF MS protocol standardization resulted in correct identification of both *C. lectularius* (London) and *C. hemipterus* (Kenya) spectra, with LSVs ranging from 1.818 to 2.415 (average: 2.112) and 2.142 to 2.849 (average: 2.574) respectively.

Blind test analysis of the wild and laboratory specimens of bedbugs used for MALDI-TOF MS protocol validation resulted in correct identification of all specimens of *C. lectularius* and *C. hemipterus* bedbugs to the species level. All wild and laboratory *C. hemipterus* specimens were properly identified according to their origin as *C. hemipterus* "Senegal" (16/16) and *C. hemipterus* "Kenya" (8/8). Twenty six of thirty five (74.28%) wild *C. lectularius* from Marseille were rightly identified as *C. lectularius* "Marseille". All specimens of *C. lectularius* "Sweden" (7/7) were properly identified according to their origin as *C. lectularius* "Sweden". As for *C. lectularius* "Germany" and "London" six of seven (85.71%) specimens of each were correctly identified according to their origin as *C. lectularius* "Germany" and *C. lectularius* "London" respectively. Only two specimens of *C. lectularius* "Germany" and *C. lectularius* "London" strains (one specimen of each) matched with reference spectra of the laboratory strain *C. lectularius* "Sweden" with LSVs of 2.502 and 2.275 respectively. Overall, 100% (123/123) of the specimens were correctly identified according to the species and 86.25% (69/80) were aptly identified according to their geographical origin. Results of blind test analyses and LSVs ranges are detailed in Table 2.

4.4. Molecular identification of wild bedbugs

A total of 16 wild *C. lectularius* and *C. hemipterus* specimens, including 8 specimens from each population (Marseille and Senegal), and 26 unambiguously identified reared laboratory specimens (9 *C.*

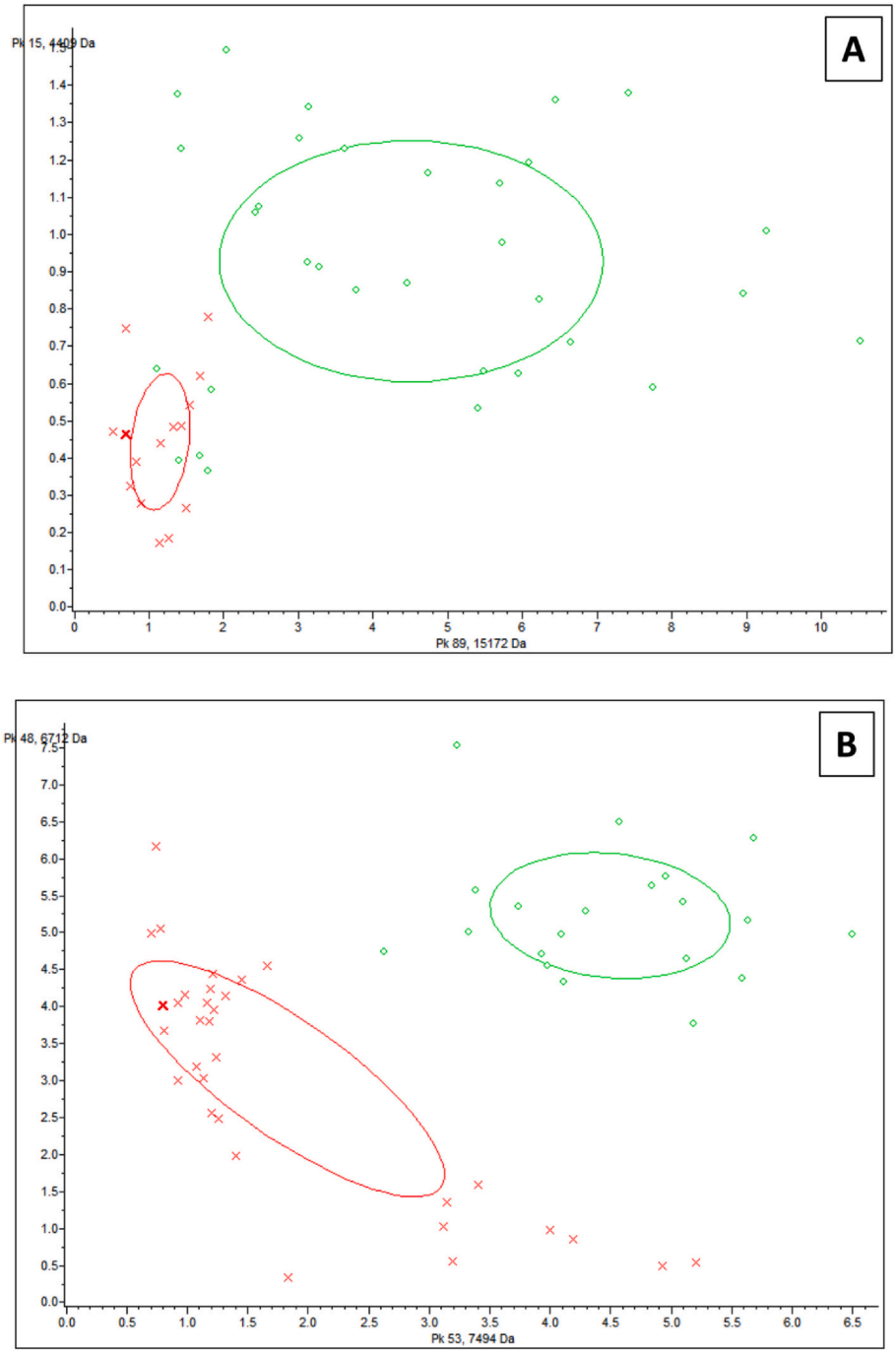

Fig. 6. MALDI-TOF MS distinction of spectra from male (circles) and female (crosses) (A) *Cimex lectularius* specimens and (B) *Cimex hemipterus* illustrated on principal component analysis using ClinProTools 2.2 software. a.u.: arbitrary units; m/z: mass-to-charge ratio.

lectularius "London" and 17 *C. hemipterus* "Kenya") from the Cimex Store (Chepstow, UK), were randomly selected for molecular identification. Good quality partial *18S* rDNA gene sequences were obtained for all tested Marseille's wild specimens (8 bedbugs) and for six of eight wild specimens from Senegal. As for laboratory specimens, six good quality partial *18S* rDNA gene sequences were obtained for *C. lectularius* (London) and eight for *C. hemipterus* (Kenya) begbugs. Good quality *COI* gene sequences were obtained for four of the eight wild *C. hemipterus* specimens from Senegal and for five of seventeen of *C. hemipterus* (Kenya) laboratory specimens. As for Marseille's wild and London's laboratory *C. lectularius* specimens, no good quality *COI* gene sequence was obtained.

BLAST analysis indicated high identity (100%) of all Marseille wild and reared *C. lectularius* consensus *18S* sequences with a reference *C. lectularius 18S* rDNA gene sequence available in Genbank (Accession Number: EU683121.1), thus confirming the morphological identification.

As for the Senegal wild and laboratory strains of *C. hemipterus*, the BLAST analysis indicated that the *18S* rDNA gene sequences matched with an identity of 99.90% and 100% query cover with a reference *C. lectularius 18S* rDNA gene sequence in Genbank (Accession Number: EU683121.1). However, the BLAST analysis of laboratory strain *C. hemipterus COI* gene sequences revealed a high identity and query cover (100% both) with reference *C. hemipterus COI* gene sequences in Genbank (Accession Numbers: MG596826.1, MG770889.1). Senegal's wild *C. hemipterus* obtained *COI* gene sequences matched with reference

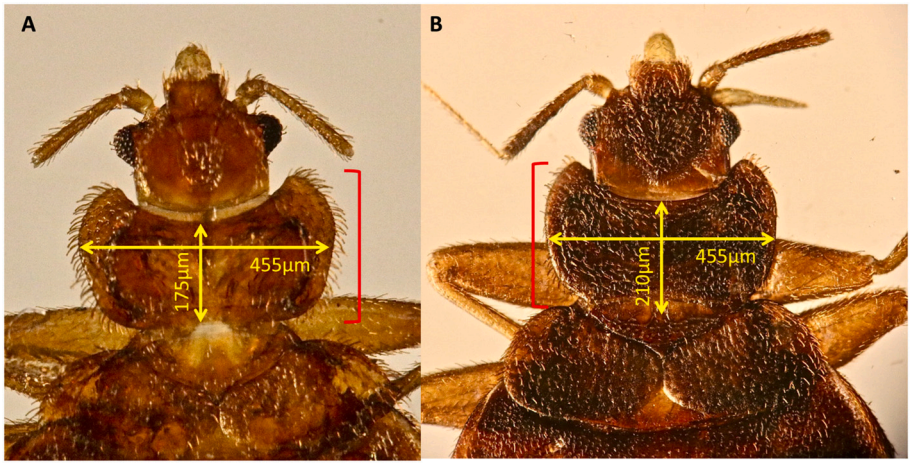

Fig. 7. Morphological differenciation of wild specimens of (A) *Cimex lectularius* and (B) *C. hemipterus* bedbugs from Marseille and Senegal respectively based on pronotum dimensions. Pronotum is indicated with red brackets. *Cimex lectularius* pronotum (A) is more than 2 ½ times as wide as long; *Cimex hemipterus* pronotum (B) is less than 2 ½ times as wide as long. Pictures were obtained on a Zeiss Axio Zoom V16 (Zeiss, Marly le Roi, France) stereomicroscope. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

C. hemipterus COI gene sequences in Genbank with an identity ranging from 99.39% to 100% and 100% query cover (Accession Number: MG770889.1, MG596826.1).

Using MEGA version X (Kumar, Stecher, Li, Knyaz, and Tamura 2018) software, a multiple sequence alignment analysis of Marseille and Senegal wild bedbugs *18S* rDNA gene sequences with unambiguously identified laboratory bedbugs (London and Kenya strains) sequences together with reference sequences of *C. lectularius* and *C. hemipterus* available in GenBank was performed, revealing a difference in one nucleotide. A "C" base in all reference, reared and wild *C. lectularius* specimens' sequences, is replaced by a "T" base in reference, reared and wild *C. hemipterus* bedbugs sequences at the same position (Supplementary Data 7). This data and *COI* gene BLAST result support the initial morphological identification despite the results of *18S* rDNA gene BLAST analysis. (All *COI* and *18S* rDNA gene FASTA sequences of the wild and laboratory tested specimens are available in Supplementary Data 3). *18S* rDNA sequences of wild and laboratory strains of *C. lectularius* (Marseille and London) have been deposited in GenBank under the following accession numbers: MN088694, MN088695 and MN088696, MN088697 respectively.

18S rDNA sequences of wild and laboratory strains of *C. hemipterus* (Senegal and Kenya) have been deposited in GenBank under the following accession numbers: MN056504, MN056505, MN056506, MN056507 and MN056500, MN056501, MN056502, MN056503 respectively. *COI* sequences of laboratory and wild strains of *C. hemipterus* (Kenya and Senegal) have been deposited in GenBank under the following accession numbers: MT423988, MT423989, MT423990, MT423991, MT423992 and MT423993, MT423994, MT423995, MT423996 respectively.

5. Discussion

There is currently an upsurge in the use of MALDI-TOF MS as a result of its performance and inexpensive operating costs. The rapid identification of microorganisms present in clinical samples has revolutionized clinical microbiology and is driving more and more laboratories towards using this approach (Lo et al., 2015, 2017; Seng et al., 2010). The supplementary use of MALDI-TOF MS facilities for medical entomology does not give rise to any additional costs, and MS identification of arthropods has already been transferred to routine practice in laboratories equipped with MS devices (Aubry et al., 2016; Sambou et al., 2015).

In this study, we propose a user-friendly, rapid and low-cost approach for the routine identification of specimens suspected to be bedbugs, which can be performed in any laboratory equipped with a MALDI-TOF MS device. Simply dissecting the head of the insect makes it possible to correctly identify the specimen to the species level, without any further entomological skills being required. Nonetheless, MALDI-TOF MS application on arthropods requires an important and tedious protocol development stage. Indeed, several parameters can influence the quality of the resulting spectrum, such as the selection of the arthropod body part, the mix volume used for protein extraction, the method and duration of grinding and the sample preservation. The different protocol combinations are compared to finally choose the one that provides the highest quality MS spectra.Once the suitable protocol is found and validated, it can be consistently and easily applied for new specimens identification (Yssouf et al., 2016). Similarly to previously published articles, several body parts have been submitted to MALDI-TOF MS analysis in order to obtain the highest quality spectra and statistical tests have been used to help select the most relevant protocol. Many reports have demonstrated the suitability of using legs for MALDI-TOF MS arthropod identification (Yssouf et al., 2016), but others have also highlighted the need to sometimes adjust the method for other body parts, as it was the case for the identification of fleas (Yssouf et al., 2014) and the detection of *Plasmodium* parasites in mosquitoes (Laroche et al., 2017a). As for bedbugs, the quality of head-derived MS spectra was greater than those obtained for legs, which are, moreover, slightly more difficult to dissect properly without experience.

Therewith, cluster analysis clearly highlighted a distinction between the two bedbug species in this study. It also allowed to distinguish between populations of *C. hemipterus* species from different geographical origins (Fig. 5). However, such specific clustering of populations of the same species has not been observed on the dendrogram for *C. lectularius* bedbugs. Nevertheless, except twelve of eighty specimens (9 *C. lectularius* "Marseille", 1 *C. lectularius* "Germany" and 1 *C. lectularius* "London") whose population has been misidentified, all the other specimens from the six tested populations were properly identified according to their geographical origin when blind tested against a database upgraded with reference spectra of *C. lectularius* (London, Germany, Sweden and Marseille) and *C. hemipterus* (Kenya and Senegal). Blind test results revealed a clear distinction between the MS profiles of laboratory and wild *C. lectularius* populations which was not noticeable in the dendrogram. This distinction between wild and laboratory reared bedbugs might highlight an environmental impact on the MS profiles. It is known that the number of reference spectra upgraded into the database has a noticeable influence on the accuracy of MALDI-TOF MS identification. The greater the number of reference spectra upgraded into the database, the more precise the identification by MALDI-TOF MS becomes (Ouarti et al., 2020). However, in our study, the number of specimens tested to check the ability of MALDI-TOF MS to differentiate specimens of the same species from different geographical origins was limited. Nevertheless, the results obtained are encouraging. It is probable that by enriching our in-lab database with

more reference spectra for each strain, we could obtain an even more precise identification.

Variations in the MS profile according to the geographical area of sampling have already been reported, particularly for mosquitoes (Raharimalala et al., 2017). Moreover, a previous study on sandflies showed, using molecular and morphometric geometric methods, the existence of an intraspecific variability within populations of *Phlebotomus sergenti* collected from different countries (Turkey, Israel, Syria and Uzbekistan). Indeed, *P. sergenti* populations clusterized separately according to their geographical origin into two distinct clades (Northeastern and Southwestern) and mitochondrial haplotypes formed three lineages with specimens from the same clade (Dvorak et al., 2011). On the other hand, in a study on the influence of environmental, spatial and temporal factors on MS spectra of ticks, the authors concluded that the variability of the MS spectra is not only linked to geographical origin, but rather to a combination of factors such as the type of habitat and the season. Indeed, the geographical provenence only seemed not to have a great impact on the MS spectra (Karger et al., 2019). Overall, the protein profiles obtained by MALDI-TOF MS are therefore influenced not only by the genetic diversity and the protein composition of the arthropod itself, but also by the proteins provided by its environment and its way of life, which are often responsible for phenotypic changes impacting its protein fingerprint, without necessarily having a genotypic origin or repercussions. A previous study on mosquitoes showed that there was a difference in the microbiota between wild and laboratory reared specimens of the same species. Wild specimen's midgut microbiota proved to be closely related to the bacterial composition and diversity of their larval gites (Tandina et al., 2016). As for *C. hemipterus* bedbugs, this population-specific clustering could be attributable to potential bedbug subpopulations, to the influence of various factors of their biotopes or even to the microbiome. Although preliminary works have discussed that dendrograms cannot be used as an additional phylogeny tool (Yssouf et al., 2013b), others have also shown that they can be very discriminative. Indeed, the two congeneric flea species *Ctenocephalides canis* and *C. felis* were successfully separated on a dendrogram (Zurita et al., 2018) as well as the two complex species *Rhodnius prolixus* and *Rh. robustus* (Laroche et al., 2017c). Previous studies have already demonstrated the ability of MALDI-TOF MS to distinguish between infected and uninfected arthropods (El Hamzaoui et al., 2018; Fotso Fotso et al., 2014; Laroche et al., 2017a; Tahir et al., 2017; Yssouf et al., 2015a, 2015b). It has also been reported that the microbiota of laboratory-reared arthropods may be significantly different to that of a wild arthropod from the same species (Tandina et al., 2016). Finally, insecticide resistance has been shown to modulate protein expression in the malaria vector *Anopheles stephensi* (Vijay et al., 2015). Variations in the resistance profile of the bedbug strain could be associated with specific MS spectra. The MALDI-TOF MS profiling algorithm is based on the comparison of protein fingerprints and this approach does not allow the accurate identification of each biomolecule involved in the MS spectra. To identify these molecules, other proteomic approaches are needed (Karger et al., 2019). Therefore, it is intricate to know if the variability observed in the MS spectra is owed to the presence of biomolecules derived from environmental factors or if this is due to protein changes subsequently to a genetic mutation. Thus, it is important to specify that MALDI-TOF MS is a proteomic tool which, cannot replace phylogenetic studies, nevertheless, it can be used as a complementary tool. Further investigations are needed to decipher the origin of the distinction between the different strains of *Cimex* species. It would also be interesting to compare MALDI-TOF MS profiles of sympatric populations from the same species. However, previous MALDI-TOF MS studies have shown that new specimens were perfectly identified using reference spectra from the same country or area (Yssouf et al., 2016). This indicates strong reproducibility of spectra from sympatric specimens. In this study we were not able to perform such a comparison for the reason that we did not have field sympatric populations, we only had one population of each origin at our disposal.

Approximately 85.63% of the spectra were included in further analyses. At this stage of the proof-of-concept, only the highest quality spectra are selected in order to assess the performance of the tool in a blind test analysis. This step is crucial for validation of the reference spectra and confirmation of the quality of the queried spectra. All these spectra could later be used as a reference to encompass any future diversity encountered.

The specimens tested in this work were fresh or freezed at −20 °C. To be applied on otherwise preserved specimens, the protocol may require some adjustments as it was the case for fleas preserved in alcohol (Zurita et al., 2018). Further studies are needed to verify the reliability of the validated protocol when it is applied to bed bugs kept in alcohol or in silica gel.

Evaluating the performance of this new tool on *C. lectularius* and *C. hemipterus* species addresses the question of the reliability of the identification for related species, making this approach relevant for vector surveillance and vector control.

In reality, although *C. lectularius* and *C. hemipterus* are the most commonly found bedbugs in human dwellings (Cannet et al., 2015), other *Cimex* species may also be in contact with humans. Bats, which can often take refuge in buildings and attics, are known to be infested with at least three *Cimex* species, *C. adjunctus* (McKee et al., 2018), *C. pipistrelli* (Bartonička and Růžičková, 2012) and *C. pilosellus* (Pearce and O'Shea, 2007). It could easily be hypothesized that these insects may come into contact with humans, and even bit them. Several studies have highlighted the role of bats as a reservoir for several viral, parasitic and bacterial pathogens (Mühldorfer, 2013). Some of these microorganisms have also been detected in their associated ectoparasites, including *Cimex* bat bugs (McKee et al., 2018). Although transmission has not yet been demonstrated, the presence of potentially infected hematophagous arthropods in human dwellings should not be overlooked. Currently, limited data is available on the occurrence of cohabitation between several *Cimex* species in the same dwelling. Further studies are needed to complete what is known about the sympatry of *Cimex* species. In our study, we only evaluated the ability of MALDI-TOF MS to differentiate between *C. lectularius* and *C. hemipterus*. These two species are very closely related and produce hybrids by interspecific mating (Doggett et al., 2012). Their clear distinction by mass spectrometry suggests that other *Cimex* species could also be specifically identified. To support this hypothesis, previously published studies on the MALDI-TOF MS identification of triatomines, which are, like bedbugs, heteropterans, have demonstrated that this tool was suitable for the identification of closely-related heteropterans, even species belonging to the same complex (Laroche et al., 2017c).

In order to confirm the species assignation of wild collected bedbugs, randomly selected specimens of both origins (Marseille and Senegal), were submitted to sequencing analysis targeting a fragment of *18S* rDNA gene and *COI* gene. Unequivocally identified laboratory specimens of both species were used as controls. Morphological, molecular and MALDI-TOF MS identification were concordant for field specimens from Marseille. The specimens were therefore, clearly assigned as *C. lectularius*. As for wild bedbugs collected in Senegal, the morphological and *18S* rDNA gene BLAST analysis were discordant. These specimens, morphologically designated as *C. hemipterus* by an entomology expert, matched with a *C. lectularius* reference sequence present in GenBank with an identity percentage of 99.90%. However, multiple sequence alignment analysis results of wild bedbugs *18S* sequences with reference and laboratory specimen sequences highlighted that sequences from Senegal wild bedbugs were identical to those of *C. hemipterus* laboratory bedbugs. Moreover, sequence alignment analysis revealed that all of the three *18S* reference sequences available in GenBank for *C. hemipterus* have gaps of approximately 50 nucleotides (Supplementary Data 7). The insufficient quality of the *18S* reference sequences for *C. hemipterus* currently deposited in GenBank, explain the very low query cover (89%) obtained in BLAST analysis compared to that obtained with *C. lectularius* reference sequence (100%). BLAST

analysis of *COI* obtained sequences allowed to confirm species assignation of Senegal's wild bedbugs specimens as *C. hemipterus* which is concordant with morphological and MALDI-TOF MS identification.

The example of the ambiguous BLAST results obtained for the *18S* rDNA gene, which is usually used for the molecular identification of hemipterans (Weirauch and Munro, 2009), and the poor quality of the reference sequences available in GenBank for *C. hemipterus*, illustrates the difficulty and the limititations of arthropods molecular identification. Correct molecular identification is conditioned by the choice of the appropriate target gene and the availability of good quality reference sequences in Genbank.

The kappa degree of agreement could be a good indicator of the concordance between the three methods used for bedbug identification (morphology, MALDI-TOF MS and molecular biology). However, since in this study these three identification methods were concordant with an agreement of 100%, the kappa coefficient is therefore incalculable in this case.

As for vector control, studies have reported a difference of susceptibility to insecticides between *C. lectularius* and *C. hemipterus*. Indeed, Fenitrothion (IUPAC name: *O*,*O*-Dimethyl O-(3-methyl-4-nitrophenyl) phosphorothioate), a widely-used phosphorothioate insecticide, has shown a considerably lower LT_{50} (median lethal time) for *C. hemipterus* than for *C. lectularius* (Tawatsin et al., 2011). A good knowledge of the susceptibility of bedbug species to a specific insecticide will make it possible to better adjust the duration of insecticidal treatments, preventing over-exposure to toxic components or, on the contrary, preventing treatments from being too short to be efficient. This strategy requires an unequivocal identification of *Cimex* specimens, which can be provided by MALDI-TOF MS, at least for the two species studied. The robustness of MALDI-TOF MS identification is based on the comprehensiveness of the reference database. Other *Cimex* species could potentially be submitted for MALDI-TOF MS analysis in further studies in order to confirm that all species can indeed clearly be distinguished using this tool. Although *Cimex* species tend to be designated depending on their geographical origin or their host, global exchange and urbanization have changed several ecological parameters. Deforestation has brought bats increasingly closer to human habitations, and bat-related touristic activities such as visiting bat caves or watching thousands of bats fly off the ground are gaining in popularity (Field, 2009). As for *C. hemipterus,* known as the tropical bedbug, it is now increasingly found in sympatry with *C. lectularius,* in Australia and Florida for example (Dang et al., 2017), or in non-tropical areas such as France and Russia (personal data). All these modifications highlight the fact that tools are needed to identify bedbugs, since the most obvious identification may not always be correct.

Although geographic origin is known to introduce slight changes in spectral profiles, it is also shown that it does not impair MALDI-TOF MS identification (Fall et al., under revision in Am. J. Trop. Med. Hyg). The major differences in the spectra, the highly specific MS identification and the morphological identification supported by molecular identification based on two genes confirm that the distinction observed using MALDI-TOF MS is based on the presence of two *Cimex* species. Albeit these results are highly encouraging, considering the similarity between *C. lectularius* and *C. hemipterus,* the robustness of the tool should be reinforced by submitting other bedbug species for MALDI-TOF MS analysis.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2020.104536.

Authors' contributions

Conceptualization: ML and PP; Data curation: ML, LB, GG and BEH; Formal analysis: ML, LB and GG; Funding acquisition: PP; Investigation: LB, GG, BEH and ML; Methodology: ML,LB, GG and BEH; Project administration: PP, ML; Resources: JMB and LB; Software: ML and LB; Supervision: ML and PP; Validation: ML; Writing - original draft: LB and ML; Writing - review & editing: LB, ML and PP. All authors reviewed and approved the final version.

Data availability

All data regarding this study is included in the manuscript and its supplementary data.

18S rDNA sequences of wild and laboratory strains of *Cimex lectularius* (Marseille and London) have been deposited in GenBank under the following accession numbers: MN088694, MN088695 and MN088696, MN088697 respectively.

18S rDNA sequences of wild and laboratory strains of *Cimex hemipterus* (Senegal and Kenya) have been deposited in GenBank under the following accession numbers: MN056504, MN056505, MN056506, MN056507 and MN056500, MN056501, MN056502, MN056503 respectively.

COI sequences of laboratory and wild strains of *C. hemipterus* (Kenya and Senegal) have been deposited in GenBank under the following accession numbers: MT423988, MT423989, MT423990, MT423991, MT423992 and MT423993, MT423994, MT423995, MT423996 respectively.

The FASTA sequences of all sequenced specimens are available as supplementary data.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

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References

Aigbodion, F.I., Megbuwe, P., 2008. Resting site preferences of cimex hemipterus (Heteroptera, Cimicidae) in human dwelling in Benin City, Nigeria. Int. J. Biomed. Heal. Sci. 4, 19–23.

Akhoundi, M., Sereno, D., Durand, R., Mirzaei, A., Bruel, C., Delaunay, P., Marty, P., Izri, A., 2020. Bed bugs (Hemiptera, Cimicidae): overview of classification. Evol. Disp. 17, 20. https://doi.org/10.3390/ijerph17124576.

Arfuso, F., Gaglio, G., Maria, J., Caracappa, G., Lupia, A., Napoli, E., Giarratana, F., Latrofa, M.S., Otranto, D., Brianti, E., 2019. Identification of phlebotomine sand flies through MALDI-TOF mass spectrometry and in- house reference database. Acta Trop. 194, 47–52. https://doi.org/10.1016/j.actatropica.2019.03.015.

Ashcroft, R., Seko, Y., Chan, L.F., Dere, J., Kim, J., McKenzie, K., 2015. The mental health impact of bed bug infestations: a scoping review. Int. J. Public Health 60 (7), 827–837. https://doi.org/10.1007/s00038-015-0713-8.

Aubry, C., Socolovschi, C., Raoult, D., Parola, P., 2016. Bacterial agents in 248 ticks removed from people from 2002 to 2013. Ticks Tick. Borne. Dis. 7 (3), 475–481. https://doi.org/10.1016/j.ttbdis.2016.02.003.

Bartonička, T., Růžičková, L., 2012. Bat bugs (Cimex pipistrelli) and their impact on non-dwelling bats. Parasitol. Res. 111 (3), 1233–1238. https://doi.org/10.1007/s00436-012-2957-z.

Berenger, J.-M., Pluot-Sigwalt, D., 2017. Présence en France de la Punaise de lit tropicale, Cimex hemipterus (Fabricius, 1803) (Hemiptera, Heteroptera, Cimicidae). Bull. la Société Entomol. Fr. 122, 423–427.

Boucheikhchoukh, M., Laroche, M., Aouadi, A., Dib, L., Benakhla, A., Raoult, D., Parola, P., 2018. MALDI-TOF MS identification of ticks of domestic and wild animals in Algeria and molecular detection of associated microorganisms. Comp. Immunol. Microbiol. Infect. Dis. 57, 39–49. https://doi.org/10.1016/j.cimid.2018.05.002.

Burgess, I., 2003. Bugs of the past—or are they on the up? Prof. Pest Controll. 32, 16–17.

Campbell, B.E., Koehler, P.G., Buss, L.J., Baldwin, R.W., 2016. Recent documentation of the tropical bed bug (Hemiptera : Cimicidae) in Florida since the common bed bug resurgence recent documentation of the tropical bed bug (Hemiptera: Cimicidae) in Florida since the common bed bug resurgence. Florida Entomol. 99, 549–551. https://doi.org/10.1653/024.099.0333.

Cannet, A., Akhoundi, M., Berenger, J.-M., Michel, G., Marty, P., Delaunay, P., 2015. A

review of data on laboratory colonies of bed bugs (Cimicidae), an insect of emerging medical relevance. Parasite 22 (21). https://doi.org/10.1051/parasite/2015021.
Chavy, A., Nabet, C., Normand, A.C., Kocher, A., Ginouves, M., Prévot, G., Vasconcelos dos Santos, T., Demar, M., Piarroux, R., de Thoisy, B., 2019. Identification of French Guiana sand flies using MALDI-TOF mass spectrometry with a new mass spectra library. PLoS Negl. Trop. Dis. 13 (2), e0007031. https://doi.org/10.1371/journal.pntd.0007031.
Dang, K., Doggett, S.L., Veera Singham, G., Lee, C.Y., 2017. Insecticide resistance and resistance mechanisms in bed bugs, Cimex spp. (Hemiptera: Cimicidae). Parasit. Vectors 10 (1), 1–31. https://doi.org/10.1186/s13071-017-2232-3.
Delaunay, P., Pharm, D., 2012. Human travel and traveling bedbugs. J. Travel Med. 19 (6), 373–379. https://doi.org/10.1111/j.1708-8305.2012.00653.x.
Delaunay, P., Blanc, V., Dandine, M., Del Giudice, P., Franc, M., Pomares-Estran, C., Marty, P., Chosidow, O., 2009. Bedbugs and healthcare- associated dermatitis, France. Emerg. Infect. Dis. 15 (6), 989–990. https://doi.org/10.1086/512370.
Delaunay, P., Blanc, V., Del Giudice, P., Levy-Bencheton, A., Chosidow, O., Marty, P., Brouqui, P., 2011. Bedbugs and infectious diseases. Clin. Infect. Dis. 52 (2), 200–210. https://doi.org/10.1093/cid/ciq102.
DeShazo, R.D., Feldlaufer, M.F., Mihm, M.C., Goddard, J., 2012. Bullous reactions to bedbug bites reflect cutaneous vasculitis. Am. J. Med. 125 (7), 688–694. https://doi.org/10.1016/j.amjmed.2011.11.020.
Doggett, S.L., Geary, M.J., Crowe, W.J., Wilson, P., Russell, R.C., 2003. Has the tropical bed bug, Cimex hemipterus (Hemiptera: Cimicidae), invaded Australia? Environ. Health 3, 80–82.
Doggett, S.L., Dwyer, D.E., Peñas, P.F., Russell, R.C., 2012. Bed bugs: clinical relevance and control options. Clin. Microbiol. Rev. 25 (1), 164–192. https://doi.org/10.1128/CMR.05015-11.
Dvorak, V., Votypka, J., Aytekin, A.M., Alten, B., Volf, P., 2011. Intraspecific variability of natural populations of Phlebotomus sergenti, the main vector of Leishmania tropica. J. Vector Ecol. 36, 49–57.
El Hamzaoui, B., Laroche, M., Almeras, L., Bérenger, J.M., Raoult, D., Parola, P., 2018. Detection of Bartonella spp. in fleas by MALDI-TOF MS. PLoS Negl. Trop. Dis. 12 (2), 1–14. https://doi.org/10.1371/journal.pntd.0006189.
El Hamzaoui, B., Laroche, M., Bechah, Y., Berenger, J.M., Parola, P., 2019. Testing the competence of *Cimex lectularius* bed bugs for the transmission of Borrelia recurrentis, the agent of relapsing fever. Am. J. Trop. Med. Hyg. 100 (6), 1407–1412. https://doi.org/10.4269/ajtmh.18-0804.
Field, H.E., 2009. Bats and emerging zoonoses: henipaviruses and SARS. Zoonoses Public Health 56, 278–284. https://doi.org/10.1111/j.1863-2378.2008.01218.x.
Folmer, O., Black, M., Hoeh, W., Lutz, R., Vrijenhoek, R., 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol. Mar. Biol. Biotechnol. 3 (5), 294–299.
Fotso Fotso, A., Mediannikov, O., Diatta, G., Almeras, L., Flaudrops, C., Parola, P., Drancourt, M., 2014. MALDI-TOF mass spectrometry detection of pathogens in vectors: the Borrelia crocidurae/Ornithodoros sonrai paradigm. PLoS Negl. Trop. Dis. 8 (7), 1–6. https://doi.org/10.1371/journal.pntd.0002984.
Gapon, D.A., 2016. First records of the tropical bed bug Cimex hemipterus (Heteroptera: Cimicidae) from Russia. Russian Acad. Sci. 25, 239–242. https://doi.org/10.31610/zsr/2016.25.2.239.
Gbakima, A., Terry, B., Kanja, F., Kortequee, S., Dukuley, I., Sahr, F., 2002. High prevalence of bedbugs Cimex hemipterus and Cimex lectularis in camps for internally displaced persons in Freetown, Sierra Leone: a pilot humanitarian investigation. West Afr. J. Med. 21, 268–271. https://doi.org/10.4314/wajm.v21i4.27994.
Halada, P., Hlavackova, K., Dvorak, V., Volf, P., 2018a. Identification of immature stages of phlebotomine sand flies using MALDI-TOF MS and mapping of mass spectra during sand fly life cycle. Insect Biochem. Mol. Biol. 93, 47–56. https://doi.org/10.1016/j.ibmb.2017.12.005.
Halada, P., Hlavackova, K., Risueño, J., Berriatua, E., Volf, P., Dvorak, V., 2018b. Effect of trapping method on species identification of phlebotomine sandflies by MALDI-TOF MS protein profiling. Med. Vet. Entomol. 32 (3), 388–392. https://doi.org/10.1111/mve.12305.
Hoppenheit, A., Murugaiyan, J., Bauer, B., Steuber, S., Clausen, P.H., Roesler, U., 2013. Identification of tsetse (Glossina spp.) using matrix-assisted laser desorption/ionisation time of flight mass spectrometry. PLoS Negl. Trop. Dis. 7 (7), 1–9. https://doi.org/10.1371/journal.pntd.0002305.
Karger, A., Bettin, B., Id, J.M.G., Id, C.K., 2019. Whole animal matrix-assisted laser desorption/ionization time-of-flight (MALDI- TOF) mass spectrometry of ticks – Are spectra of Ixodes ricinus nymphs influenced by environmental, spatial, and temporal factors? PLoS One 14 (1), 1–10. https://doi.org/10.1371/journal.pone.0210590.
Kassianoff, L., 1937. Étude morphologique et biologique de la famille des cimicidés. In: Masson (Ed.), Annales de Parasitologie Humaine et Comparée, Tome XV. Masson et cie, pp. 97–124.
Kaufmann, C., Ziegler, D., Schaffner, F., Carpenter, S., Pfluger, V., Mathis, A., 2011. Evaluation of matrix-assisted laser desorption/ionization time of flight mass spectrometry for characterization of Culicoides nubeculosus biting midges. Med. Vet. Entomol. 32–38. https://doi.org/10.1111/j.1365-2915.2010.00927.x.
Kaufmann, C., Schaffner, F., Ziegler, D., Pflüger, V., Mathis, A., 2012. Identification of field-caught Culicoides biting midges using matrix-assisted laser desorption/ionization time of flight mass spectrometry. Parasitology 139, 248–258. https://doi.org/10.1017/S0031182011001764.
Kweka, E.J., Mwang'onde, B.J., Kimaro, E.E., Msangi, S., Tenu, F., Mahande, A.M., 2009. Insecticides susceptibility status of the bedbugs (*Cimex lectularius*) in a rural area of Magugu, northern Tanzania. J. Global Infect. Dis. 1, 102–106. https://doi.org/10.4103/0974-777X.56252.
Lafri, I., Almeras, L., Bitam, I., Caputo, A., Yssouf, A., 2016. Identification of Algerian Field-Caught Phlebotomine Sand Fly Vectors by MALDI- TOF MS 1–19. https://doi.org/10.1371/journal.pntd.0004351.
Laroche, M., Almeras, L., Pecchi, E., Bechah, Y., Raoult, D., Viola, A., Parola, P., 2017a. MALDI-TOF MS as an innovative tool for detection of Plasmodium parasites in Anopheles mosquitoes. Malar. J. 16 (1), 1–10. https://doi.org/10.1186/s12936-016-1657-z.
Laroche, M., Bérenger, J.M., Delaunay, P., Charrel, R., Pradines, B., Berger, F., Ranque, S., Bitam, I., Davoust, B., Raoult, D., Parola, P., 2017b. Medical entomology: a Reemerging Field of research to better understand vector-borne infectious diseases. Clin. Infect. Dis. 65, S30–S38. https://doi.org/10.1093/cid/cix463.
Laroche, M., Bérenger, J.M., Gazelle, G., Blanchet, D., Raoult, D., Parola, P., 2017c. MALDI-TOF MS protein profiling for the rapid identification of Chagas disease triatomine vectors and application to the triatomine fauna of French Guiana. Parasitology 145 (5), 665–675. https://doi.org/10.1017/S0031182017001342.
Lawrence, A.L., Batovska, J., Webb, C.E., Lynch, S.E., Blacket, M.J., Šlapeta, J., Parola, P., Laroche, M., 2018. Accurate identification of Australian mosquitoes using protein profiling. Parasitology 146 (4), 462–471. https://doi.org/10.1017/S0031182018001658.
Lederer, G., 1950. Auftreten von Cimex hemipterus Fabricius 1803 = *C. rotundatus* Sign. sowie anderer Cimexarten in Hessen (Heteropt. Cimicidae). J. Pest. Sci. 2004 (23), 44–46. https://doi.org/10.1007/BF02044192.
Leulmi, H., Bitam, I., Berenger, J.M., Lepidi, H., Rolain, J.M., Almeras, L., Raoult, D., Parola, P., 2015. Competence of *Cimex lectularius* bed bugs for the transmission of Bartonella quintana, the agent of trench fever. PLoS Negl. Trop. Dis. 9 (5), 4–15. https://doi.org/10.1371/journal.pntd.0003789.
Lin, Z., Cai, Z., 2018. Negative ion laser desorption/ionization time-of-flight mass spectrometric analysis of small molecules by using nanostructured substrate as matrices. Mass Spectrom. Rev. 37 (5), 681–696. https://doi.org/10.1002/mas.21558.
Lo, C.I., Fall, B., Sambe-Ba, B., Diawara, S., Gueye, M.W., Mediannikov, O., Sokhna, C., Faye, N., Diemé, Y., Wade, B., Raoult, D., Fenollar, F., 2015. MALDI-TOF mass spectrometry: a powerful tool for clinical microbiology at Hôpital principal de Dakar, Senegal (West Africa). PLoS One 10 (12), 1–12. https://doi.org/10.1371/journal.pone.0145889.
Lo, C., Fall, B., Samb-Ba, B., Flaudrops, C., Faye, N., Mediannikov, O., Sokhna, C., Wade, B., Raoult, D., Fenollar, F., 2017. Value of matrix assisted laser desorption ionizationtime of flight (MALDI-TOF) mass spectrometry in clinical microbiology and infectious diseases in Africa and tropical areas. Afr. J. Microbiol. Res. 11, 1360–1370. https://doi.org/10.5897/AJMR2016.8181.
Masini, P., Zampetti, S., MiñónLlera, G., Biancolini, F., Moretta, I., Romani, R., Tramontana, M., Hansel, K., Stingeni, L., 2019. Infestation by the tropical bedbug Cimex hemipterus (Hemiptera: Cimicidae): first report in Italy. J. Eur. Acad. Dermatol. Venereol. 34, 28–30. https://doi.org/10.1111/jdv.15876.
Mathis, A., Depaquit, J., Dvořák, V., Tuten, H., Bañuls, A.L., Halada, P., Zapata, S., Lehrter, V., Hlavačková, K., Prudhomme, J., Volf, P., Sereno, D., Kaufmann, C., Pflüger, V., Schaffner, F., 2015. Identification of phlebotomine sand flies using one MALDI-TOF MS reference database and two mass spectrometer systems. Parasit. Vectors 8 (1), 1–9. https://doi.org/10.1186/s13071-015-0878-2.
McKee, C.D., Osikowicz, L.M., Schwedhelm, T.R., Bai, Y., Castle, K.T., Kosoy, M.Y., 2018. Survey of parasitic Bacteria in bat bugs, Colorado. J. Med. Entomol. 55 (1), 237–241. https://doi.org/10.1093/jme/tjx155.
Mewara, A., Sharma, M., Kaura, T., Zaman, K., Yadav, R., Sehgal, R., 2018. Rapid identification of medically important mosquitoes by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Parasit. Vectors 11 (281), 1–9. https://doi.org/10.1186/s13071-018-2854-0.
Mühldorfer, K., 2013. Bats and bacterial pathogens: a review. Zoonoses Public Health 60 (1), 93–103. https://doi.org/10.1111/j.1863-2378.2012.01536.x.
Myamba, J., Maxwell, C., Asidi, A., Curtis, C., 2002. Pyrethroid resistance in tropical bedbugs, Cimex hemipterus, associated with use of treated bednets. Med. Vet. Entomol. 16, 448–451.
Nascimento, G.L.G., 2010. Investigação da ocorrência de infestação por Cimicidae (Heteroptera: Cimicomorpha) na Região Metropolitana de São Paulo, no período de 2004 a 2009. (São Paulo).
Newberry, K., Mchunu, Z.M., 1989. Changes in the relative frequency of occurrence of infestations of two sympatric species of bedbug in northern Natal and KwaZulu, South Africa. Trans. R. Soc. Trop. Med. Hyg. 83, 262–264.
Onah Isegbe, E., Alu, C., Omudu, A.E., 2014. A survey of bedbug (*Cimex lectularius*) infestation in some homes and hostels in Gboko, Benue state, Nigeria. Psyche (Stuttg) 2014, 1–5. https://doi.org/10.1155/2014/762704.
Ouarti, B., Laroche, M., Righi, S., Meguini, M.N., Benakhla, A., Raoult, D., Parola, P., 2020. Development of MALDI-TOF mass spectrometry for the identification of lice isolated from farm animals. Parasite 28.
Patel, D., Elston, D.M., 2012. What's eating you? Bedbugs revisited (*Cimex lectularius*). Cutis 90 (4), 173–175.
Pearce, R.D., O'Shea, T.J., 2007. Ectoparasites in an urban population of big Brown bats (*Eptesicus Fuscus*) in Colorado. J. Parasitol. 93 (3), 518–530. https://doi.org/10.1645/ge-973r.1.
Péricart, J., 1973. Hémiptères Anthocoridae, Cimicidae et Microphysidae de l'Ouest paléarctique. In: Faune de l'Europe et du bassin méditerranéen. Bulletin de la Société entomologique de France.
Persson Vinnersten, T., 2017. Tropisk vägglus Cimex hemipterus (Fabricius, 1803) en etablerad vägglusart i Sverige (Heteroptera: Cimicidae). Entomol. Tidskr. 138, 67–70.
Raharimalala, F.N., Andrianinarivomanana, T.M., Rakotondrasoa, A., Collard, J.M., Boyer, S., 2017. Usefulness and accuracy of MALDI-TOF mass spectrometry as a supplementary tool to identify mosquito vector species and to invest in development of international database. Med. Vet. Entomol. 31 (3), 289–298. https://doi.org/10.1111/mve.12230.
Rosen, S., Hadani, A., Gur Lavi, A., Berman, E., Bendheim, U., Hisham, A.Y., 1987. The

occurrence of the tropical bedbug (Cimex hemipterus, fabricius) in poultry barns in Israel. Avian Pathol. 16, 339–342. https://doi.org/10.1080/03079458708436381.
Rothen, J., Githaka, N., Kanduma, E.G., Olds, C., Pflüger, V., Mwaura, S., Bishop, R.P., Daubenberger, C., 2016. Matrix-assisted laser desorption/ionization time of flight mass spectrometry for comprehensive indexing of east African ixodid tick species. Parasit. Vectors 9 (151), 1–15. https://doi.org/10.1186/s13071-016-1424-6.
Salazar, R., Castillo-Neyra, R., Tustin, A.W., Borrini-Mayorí, K., Náquira, C., Levy, M.Z., 2015. Bed bugs (*Cimex lectularius*) as vectors of Trypanosoma cruzi. Am. J. Trop. Med. Hyg. 92 (2), 331–335. https://doi.org/10.4269/ajtmh.14-0483.
Sambou, M., Aubadie-Ladrix, M., Fenollar, F., Fall, B., Bassene, H., Almeras, L., Sambe-Ba, B., Perrot, N., Chatellier, S., Faye, N., Parola, P., Wade, B., Raoult, D., Mediannikov, O., 2015. Comparison of matrix-assisted laser desorption ionization-time of flight mass spectrometry and molecular biology techniques for identification of culicoides (diptera: Ceratopogonidae) biting midges in Senegal. J. Clin. Microbiol. 53 (2), 410–418. https://doi.org/10.1128/JCM.01855-14.
Schaffner, F., Kaufmann, C., Pflüger, V., Mathis, A., 2014. Rapid protein profiling facilitates surveillance of invasive mosquito species. Parasit. Vectors 7, 1–7. https://doi.org/10.1186/1756-3305-7-142.
Seng, P., Rolain, J.-M., Fournier, P.E., La Scola, B., Drancourt, M., Raoult, D., 2010. MALDI-TOF-mass spectrometry applications in clinical microbiology. Future Microbiol. 5 (11), 1733–1754. https://doi.org/10.1016/s0140-6736(01)91276-0.
Simonetti, V., Strippoli, D., Pinciara, B., Spreafico, A., Motolese, A., 2008. Ekbom syndrome: a disease between dermatology and psychiatry. G. Ital. Dermatol. Venereol. 143 (6), 415–419.
Tahir, D., Socolovschi, C., Marié, J. Lou, Ganay, G., Berenger, J.M., Bompar, J.M., Blanchet, D., Cheuret, M., Mediannikov, O., Raoult, D., Davoust, B., Parola, P., 2016. New rickettsia species in soft ticks Ornithodoros hasei collected from bats in French Guiana. Ticks Tick. Borne. Dis. 7 (6), 1089–1096. https://doi.org/10.1016/j.ttbdis.2016.09.004.
Tahir, D., Almeras, L., Varloud, M., Raoult, D., Davoust, B., Parola, P., 2017. Assessment of MALDI-TOF mass spectrometry for filariae detection in *Aedes aegypti* mosquitoes. PLoS Negl. Trop. Dis. 11 (12), 1–18. https://doi.org/10.1371/journal.pntd.0006093.
Tandina, F., Almeras, L., Koné, A.K., Doumbo, O.K., Raoult, D., Parola, P., 2016. Use of MALDI-TOF MS and culturomics to identify mosquitoes and their midgut microbiota. Parasit. Vectors 9 (1), 1–11. https://doi.org/10.1186/s13071-016-1776-y.
Tawatsin, A., Thavara, U., Chompoosri, J., Phusup, Y., Jonjang, N., Khumsawads, C., Bhakdeenuan, P., Sawanpanyalert, P., Asavadachanukorn, P., Mulla, M.S., Siriyasatien, P., Debboun, M., 2011. Insecticide resistance in bedbugs in Thailand and laboratory evaluation of insecticides for the control of *Cimex hemipterus* and *Cimex lectularius* (Hemiptera: Cimicidae). J. Med. Entomol. 48, 1023–1030. https://doi.org/10.1603/me11003.
Usinger, R.L., 1966. In: Thomas (Ed.), Monograph of Cimicidae (Hemiptera – Heteroptera). Entomological Society of America.
Venkatachalam, P., Beldavady, B., 1962. Loss of hemoglobin iron due to excessive biting by bedbugs. R. Soc. Trop. Med. Hyg. 56, 218–221.
Vijay, S., Rawal, R., Kadian, K., Raghavendra, K., Sharma, A., 2015. Annotated differentially expressed salivary proteins of susceptible and insecticide- resistant mosquitoes of Anopheles stephensi. PLoS One 10 (3), 1–15. https://doi.org/10.1371/journal.pone.0119666.
Wang, L., Xu, Y., Zeng, L., 2013. Resurgence of bed bugs (Hemiptera: Cimicidae) in mainland China. Florida Entomol. 96, 131–136.
Weirauch, C., Munro, J.B., 2009. Molecular phylogeny of the assassin bugs (Hemiptera: Reduviidae), based on mitochondrial and nuclear ribosomal genes. Mol. Phylogenet. Evol. 53 (1), 287–299. https://doi.org/10.1016/j.ympev.2009.05.039.
Yssouf, A., Flaudrops, C., Drali, R., Kernif, T., Socolovschi, C., Berenger, J.M., Raoult, D., Parola, P., 2013a. Matrix-assisted laser desorption ionization-time of flight mass spectrometry for rapid identification of tick vectors. J. Clin. Microbiol. 51 (2), 522–528. https://doi.org/10.1128/JCM.02665-12.
Yssouf, A., Socolovschi, C., Flaudrops, C., Ndiath, M.O., Sougoufara, S., Dehecq, J.S., Lacour, G., Berenger, J.M., Sokhna, C.S., Raoult, D., Parola, P., 2013b. Matrix-assisted laser desorption ionization - time of flight mass spectrometry: an emerging tool for the rapid identification of mosquito vectors. PLoS One 8 (8), 1–10. https://doi.org/10.1371/journal.pone.0072380.
Yssouf, A., Socolovschi, C., Leulmi, H., Kernif, T., Bitam, I., Audoly, G., Almeras, L., Raoult, D., Parola, P., 2014. Identification of flea species using MALDI-TOF/MS. Comp. Immunol. Microbiol. Infect. Dis. 37 (3), 153–157. https://doi.org/10.1016/j.cimid.2014.05.002.
Yssouf, A., Almeras, L., Berenger, J.M., Laroche, M., Raoult, D., Parola, P., 2015a. Identification of tick species and disseminate pathogen using hemolymph by MALDI-TOF MS. Ticks Tick. Borne. Dis. 6 (5), 579–586. https://doi.org/10.1016/j.ttbdis.2015.04.013.
Yssouf, A., Almeras, L., Terras, J., Socolovschi, C., Raoult, D., Parola, P., 2015b. Detection of rickettsia spp in ticks by MALDI-TOF MS. PLoS Negl. Trop. Dis. 9 (2), 1–16. https://doi.org/10.1371/journal.pntd.0003473.
Yssouf, A., Almeras, L., Raoult, D., Parola, P., 2016. Emerging tools for identification of arthropod vectors. Future Microbiol. 11 (4), 549–566. https://doi.org/10.2217/fmb.16.5.
Zhang, J., Xia, Y., Wang, C., Han, D., Ren, D., Zheng, J., Xu, X., He, Y., Wang, D., 2020. Morphological and molecular identification of tropical bed bugs from two cities of the Pearl River Delta in China. J. Med. Entomol. XX 1–4. https://doi.org/10.1093/jme/tjaa155.
Zorrilla-Vaca, A., Silva-Medina, M.M., Escandón-Vargas, K., 2015. Bedbugs, Cimex spp.: their current world resurgence and healthcare impact. Asian Pacific J. Trop. Dis. 5, 342–352. https://doi.org/10.1016/S2222-1808(14)60795-7.
Zurita, A., Djeghar, R., Callejón, R., Cutillas, C., Parola, P., Laroche, M., 2018. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry as a useful tool for the rapid identification of wild flea vectors preserved in alcohol. Med. Vet. Entomol. 33 (2), 185–194. https://doi.org/10.1111/mve.12351.