Enhanced procedures for mosquito identification by MALDI-TOF MS

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Research Article

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Abstract

Background. The increasing interest in arthropod identification by MALDI-TOF MS conducted to the emergence of heterogenic procedures, including sample preparation and selection of body parts to use. The absence of a consensual strategy hampers direct inter-studies comparisons and brings confusion to new users. Establishing optimized procedures and standardized protocols for mosquito identification by MALDI-TOF MS becomes compulsory, allowing notably the sharing of reference MS databases.

Objective. Then, here, we propose to assess the optimal conditions for mosquito identification using MALDI-TOF MS profiling.

Methods. Three homogenization methods, two manuals and one automatic, applied on three distinct body parts, legs, thorax and head, were evaluated on two mosquito laboratory strains, *Anopheles coluzzii* and *Aedes aegypti*. MS profiles reproducibility, identification rate with relevant scores and the suitable procedures for high-throughput analyses were the main criteria for establishing optimized guidelines. Additionally, the consequences of blood feeding and geographical origin were evaluated using both laboratory strain and field collected mosquitoes.

Results. The three body parts exhibited relevant score values for mosquito identification using MALDI-TOF MS profiling; however, thorax and legs appeared the most suitable, independently of homogenization methods or species. Although manual grindings displayed high identification performance for the three body parts, this homogenization mode is not adapted to process a large number of samples. Then, the automatic procedure was selected as the reference homogenization method. Blood feeding status did not hamper the identification of mosquito species, despite the presence of MS peaks from blood origin in MS profiles from three body parts tested on both species. Finally, a significant improvement in identification scores was obtained for specimens from the field when MS spectra of species from the same geographical area were added to the database.

Conclusion. The present work established guidelines concerning the selection of mosquito anatomic part and modality of sample preparation (*e.g.*, homogenization) for future specimen identification by MALDI-TOF MS profiling. These standardized operational protocols could be used as references for creating an international MS database.

Background

Mosquitoes, hematophagous dipterans from the Culicidae family, are considered among major arthropods of public health importance [1–3]. The Culicidae family encompasses about 3550 species and subspecies of 44 genera [4–6]. Among them, *Anopheles* spp., *Aedes* spp. and *Culex* spp. are the most important vectors due to their role in the transmission of a variety of pathogens, including parasites, viruses and bacteria [4, 5]. Although these mosquitoes are mainly distributed in tropical and subtropical areas [6], global warming, associated with the travel of goods and people, facilitated the colonization of new areas. Consequently, the mosquito invasion leads to the emergence of diseases where they were previously non-existent, culminating in recurrent outbreaks and pandemics. Mosquito borne diseases (MBDs) are considered a worldwide concern [7, 8].

Among the diversity of mosquito species, some are involved in MBD transmission [5, 9]. The identification of mosquito species is of prime importance for vector surveillance and control. Morphological identification keys are the most widely used technique for entomological surveillance [10, 11]. Although this laborious approach remains the primary and reference method for species identification, it requires robust entomological knowledge. A recent study revealed that only 81% and 64% of entomologists succeeded identify mosquito specimens at the genus and species level, respectively [12]. Furthermore, this technique is limited when specimens are damaged, belong to species complexes or new in a specific area [13]. To overcome these issues, the use of molecular biology tools has emerged [12]. This strategy target

genes for species identification, such as the mitochondrial cytochrome c oxidase subunit 1 (COI), the internal transcribed spacer 2 (ITS2), the intergenic spacer (IGS) or regions from ribosomal subunits [14]. Molecular tools are usually time-consuming and can be expensive, limiting their use in large scale studies [15].

In this sense, a cheaper methodology, rapid, technically reproducible and straightforward, allowing large-scale processing, has emerged as an alternative: the matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) [15]. This proteomic tool, routinely used in microbiology diagnosis laboratories for bacteria or yeast identification [16–18], has been efficiently applied in several medical entomology studies for arthropods identification, such as flies [19, 20], ticks [21, 22] or fleas [23]. This tool was also successfully used to identify mosquitoes from laboratory or field origins [24–26]. Its performances for mosquito identification were highlighted in a recent study reporting the successful distinction of members from the *Anopheles gambiae* complex, *An. gambiae*, *An. coluzzii* and *An. arabiensis* field-collected [27].

Mosquito legs were the main body part initially used for specimen identification by MALDI-TOF MS [23, 28]. An optimised procedure was established to standardize MS identification of mosquitoes for sample preparation of adult and larval stages using legs or whole specimens, respectively [29]. The performance of imago mosquito identification by MS using legs was repeatedly confirmed [26, 27, 30, 31]. However, mosquito legs present the disadvantage to be breakable, hampering MS identification of specimens that have lost their legs. To circumvent this limitation, the thoraxes were recently proposed as a complementary body part for mosquito identification by MS profiling [32]. The submission of these two body parts to MS profiling demonstrated a corroboration of species identification, reinforcing identification confidence and success rate [32, 33].

The increasing interest in mosquito identification by MALDI-TOF MS led to the emergence of heterogenic procedures, including the sample preparation mode and the body part selection [15]. Concerning body parts, some studies reported the use of thoraxes and cephalothoraxes [34, 35]. More recently, Nabet et al [24] emphasized that the mosquito head appeared to be the most appropriate body part for MS identification. In addition, methods for homogenizing the samples vary according to studies; some used automatic devices, such as TissueLyser (TL) and others ground samples manually with a micropipette (MP) [24] or pellet pestles (PP) [36]. The sample homogenization mode and quantity of mix buffer could also influence the quality of MS spectra, altering the spectral matching with reference MS spectra from the database [32, 37]. Other factors such as geographical origin [30, 37, 38] or engorgement status [24] were also reported to induce variations in the MS spectra, which could perturb species identification. The absence of a consensual strategy for mosquito identification using MALDI-TOF MS prejudice direct inter-studies comparisons, the sharing of reference MS spectra database (DB) and brings confusion to new users.

It becomes compulsory to clarify which mosquito body part to select for MS identification, the procedures for sample preparation and the effect of some endogenous and exogenous factors on these MS profiles. In this sense, the intra-species reproducibility and inter-species specificity of MS spectra from heads, thoraxes (without wings) and legs, homogenized with automatic or manual modes, were compared to determine the more suitable conditions for MS mosquito specimen identification. The impact of mosquito blood engorgement, and the geographical origin of specimens on MS profiles, were also assessed. Laboratory-reared and field specimens from the same species were used for these evaluations.

Methods

Ethical Statement

The study was conducted under the ethical clearance N° 2018/06/1036/CE/CNERSH/SP and N° 1284/CRERSHC/2021 granted by the Cameroon National (CNE) and Centre Regional (CRE) Ethics Committee for Research on Human Health.

Authorization to carry out the study was obtained from the administration and heads of household (HoH) through an inform consent form. The volunteer collectors were adults living in the collection sites. After each collection performed per human landing catch (HLC), malaria prophylaxis was given to volunteer collectors. Mosquitoes from Congo and other localities were collected at larval stages or with traps with no need for ethical authorizations. Mosquitoes from Cameroon and Congo were shipped to VITROME according to importation authorization N° ER-22-2020 and were provided by the Research Institute of Yaoundé. Eggs of mosquitoes from Brazil were kindly provided by the Oswaldo Cruz Foundation, according to the material transfer agreement and importation authorization N° ER-12-2018.

Mosquitoes

Laboratory reared and field collected mosquitoes were used in this study (Table 1). Four laboratory strains were used, *Aedes (Ae.) aegypti* (Bora), originated from French Polynesia (i.e., Bora Bora), *Ae. albopictus* (Mrs), originated from the south of France (i.e., Marseille), *Anopheles (An.) coluzzii* (Dkr), originated from Senegal (i.e., Dakar) and *An. gambiae* (Kis), from Kenya (ie, Kisumu). *Ae. aegypti* (Bora), *Ae. albopictus* (Mrs) and *An. coluzzii* (Dkr) were reared at VITROME (Vecteurs – Infections Tropicales et Méditerranée), whereas *An. gambiae* (Kis) were reared at the "Institut de Recherche de Yaoundé" (IRY). Breeding was performed under controlled conditions of temperature (28 ± 1°C), relative humidity (80 ± 10%) and photoperiod in incubators (12h:12h) (Panasonic cooled incubator) as previously described [37]. Briefly, eggs were laid to hatch in trays with dechlorinated water and larvae were fed with fish feed (JBL Novo Prawn or Tetramin®). Pupae were transferred to mosquito cages until the emergence of adults. They were fed with a 10% glucose solution. For eggs production, blood meals were conducted through a Parafilm-membrane (hemotek membrane feeding systems, Discovery Workshops, UK) using fresh heparinized human blood [37]. Only female imago mosquitoes were included in the study. The mosquitoes were stored at -20 °C until future analyses.

Field collection of mosquitoes included larval and adult specimens from *Ae. albopictus* (Cameroon) and *An. gambiae* (Cameroon and Congo). As in laboratory-reared, uniquely females were selected for this study. Mosquito details are presented in Table 1. Collected specimens were stored at -20 °C or in silicate at room temperature from a few months to 1 year. All field-collected specimens were identified morphologically under a binocular loupe (Leica M80, Leica, Nanterre, France), using morphological descriptions [9,30]. Eggs from *Ae. aegypti* (Oia) originated from Brazil (i.e., Oiapoque) were hatched at VITROME laboratory and raised until adulthood. Females were dissected (see Additional file 1) and stored at -20°C until their use.

Molecular identification of field collected mosquitoes

DNA was extracted using the QIAamp DNA tissue extraction kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The mosquitoes molecular identification followed the previously described protocols in [14] [39][40] (see Additional file 1 for details).

Preliminary tests for mosquito head homogenization

A volume of 20µL, 30µL and 40µL of mix buffer composed of a mix (50/50) of the volume of 70% (v/v) formic acid (Sigma-Aldrich, Lyon, France) and 50% (v/v) acetonitrile (Fluka, Buchs, Switzerland) were added to the 5 individuals heads of female mosquitoes per species, *Ae. aegypti* (Bora) and *An. coluzzii* (Dkr). The samples were homogenized using the automatic method described in the next section, prior submission to MALDI-TOF MS. Intensity and reproducibility of MS profiles were the criteria used to establish the optimal mix buffer volume to use for mosquito head homogenization.

Sample homogenization for MALDI-TOF MS analysis

The laboratory strains *Ae. aegypti* (Bora) and *An. coluzzii* (Dkr) were used to compare the three homogenization procedures, including the two manuals: using a micropipette (MP) or pellet pestles (PP), and one automatic, using TissueLyser (TL, Qiagen). Head, legs and thorax from ten mosquitoes per species were used for each homogenization condition. The quantity of mix buffer added for homogenization was body part dependent. Respectively, 30µL and 50 µL of mix buffer were used for legs and thoraxes, as previously described [32, 33], and 30µL of mix buffer were used for heads based on our preliminary tests. For themanual homogenization, the samples were ground either with MP (with tips of 10µl) or PP until completed homogenization as previously described [24, 41]. Automatic homogenization consisted of adding glass beads 0.1mm (BioSpec Products, Bartlesville, US) in each sample tube, followed by a homogenization cycle of 3 X 1 minute at 30 Hertz using a TissueLyser device, according to the standardized, automated setting described previously [29]. After sample homogenization, a quick spin-down centrifugation at 200× g for 1 min was performed, and 1µL of the supernatant was loaded, in quadruplicate, into the MALDI-TOF MS steel target plate (Bruker Daltonics, Wissembourg, France). The grinding time for 5 samples was recorded per homogenization method and body part to estimate the time required per method.

Mosquito engorgement

The two laboratory strains, *Ae. aegypti* (Bora) and *An. coluzzii* (Dkr), were used to assess the consequences of engorgement on respective MS profiles from heads, legs and thoraxes. Mosquitoes were engorged with human blood through the artificial feeding Hemotek membrane feeding system (Discovery Workshops), as described previously [42]. Engorged specimens were transferred to a new cage, and females were collected after 2, 6, 12, 24, 48 and 72 hours post engorgement. Twenty females were collected per time point and species, excepted at 72 hours, for which only ten were assessed. The specimens were stored at -20°C until processing. For MALDI-TOF MS analysis, each specimen was dissected, and the heads, legs and thoraxes were homogenized using the automated TissueLyser protocol described above.

Sample loading on MALDI-TOF MS target plate

One microliter of supernatant of each sample was spotted on the MALDI-TOF MS steel target plate (Bruker Daltonics) in quadruplicate and covered with 1 µL of matrix solution before submitted to Microflex LT MALDI-TOF Mass Spectrometer device (Bruker Daltonics). Details regarding sample loading, MALDI-TOF MS parameters and MS spectra analysis are presented in the Additional file 1.

Creation of reference databases and blind tests

Three databases were created for this study to assess the impact of body part selection, sample homogenization mode, engorgement or geographical origin of specimens on the accuracy of species identification (Figure 1, Table 1). The reference MS spectra were created using spectra from heads, legs and thoraxes of two specimens per species, according to Table 1, using MALDI-Biotyper software v3.0. (Bruker Daltonics)[38]. All specimens included in the databases were not engorged. MS spectra were created with an unbiased algorithm using the peak position, intensity, and frequency information. The first database (DB1) comprised MS spectra of heads, legs and thoraxes homogenized either by MP, PP or TL methods from two laboratory strains, *Ae. aegypti* (Bora) and *An. coluzzii* (Dkr). This reference MS DB was used to assess the effect of homogenization mode per body part (Figure 1A) and the impact of blood meal on MS spectra (Figure 1B). DB2 and DB3 were composed of heads, legs and thoraxes MS spectra from laboratory-reared or laboratory-reared plus field collected mosquito species, respectively (see Table 1 for details), to assess the impact of geographical origin on specimen identification.

A total of 24 unfed and 110 engorged specimens per *Ae. aegypti* (Bora) and *An. coluzzii* (Dkr) species, were tested against DB1, whereas 124 and 116 specimens from 4 distinct species, including laboratory-reared and field mosquitoes,

were used against DB2 and DB3. The reliability of species identification was estimated using the log score values (LSVs). The calculation of this score, which ranged from 0 to 3, was done using a biostatistical algorithm from the MALDI Biotyper software v.3.0. According to previous studies [33, 43, 44], LSVs greater than 1.8 were considered reliable for species identification. Data were analyzed by R software (R core Team, R Foundation for Statistical Computing, Vienna, Austria).

Statistical analysis

After verifying that the LSVs in each group (homogenization mode, body parts) did not follow a Gaussian distribution (Shapiro-Wilk test), the Kruskal-Wallis and Mann-Whitney tests were computed when appropriate using R software (R core Team, R Foundation for Statistical Computing, Vienna, Austria). Frequencies were compared by the Chi-square test. All differences were considered significant at $p < 0.05$.

Results

Quantity of mix buffer appropriate for mosquito head homogenization and MS submission

Five *Ae. aegypti* (Bora) and five *An. coluzzii* (Dkr) mosquitoes were used to determine the appropriate quantity of mix buffer to add to the mosquito head for protein extraction before sample homogenization and MALDI-TOF MS submission. The visual comparison of MS spectra according to the volume of mix buffer used indicated a high similarity per species (Figure S1A-B). The mean CCI values from mosquito head MS spectra were elevated, ranging from 0.77 to 0.84, and were not significantly different, per species, whatever the volume of mix used ($p>0.05$, Kruskal Wallis test) (Figure S1C). The analysis of the MS profiles and CCI values indicated a good reproducibility of head MS spectra independently of the mix buffer volume used for sample homogenization. Interestingly, a slight decrease in MS profile peak intensity was noticed when 40µL of mix buffer was used for both species tested (Figure S1A-B). Then, the addition of 20µL or 30µL of mix buffer seems more adapted. Here, to limit experimental variables, the volume of 30µL, as used for legs homogenization, was chosen to add to the head.

Consequences of homogenization procedures and mosquito body part on MS profiles

Heads, thoraxes (without wing) and legs from *Ae. aegypti* (Bora) and *An. coluzzii* (Dkr) were homogenized using either TL, MP or PP method prior to submission to MALDI-TOF MS. The three body parts from 10 specimens per species were tested per homogenization mode. A total of 180 samples generated 720 MS spectra with high intensity, independently of the mosquito body part and homogenization methods. The MS profiles were visually reproducible per body part for each species (Figure 2). Interestingly, the MS patterns seem also species specifics and body part specifics. Cluster analysis using two specimens per species and per homogenization method revealed that all samples from the same mosquito species clustered on the same branch (Figure 3). Samples were grouped per body part for each species, reflecting spectra reproducibility. For each body part, the intertwining of spectra, independently of the homogenization mode, underlined that the homogenization method seems not to impact MS spectra. This cluster analysis suggested that the primary determinant for the MS profiles was the species, followed by the body part, with a singularity of legs MS spectra compared to heads and thoraxes.

A CCI-based analysis confirmed the reproducibility of MS spectra per body part and per species independently of the homogenization mode (Figure 4). Effectively, the mean CCI values of each body part were comparable among homogenization mode for both species. However, higher mean CCI values were obtained for thoraxes, followed by legs and finally by heads for both species. The comparisons of mean CCI values showed significant differences between thoraxes and legs ($p<0.0001$, Mann-Whitney test), thoraxes and heads ($p<0.0001$, Mann-Whitney test), and legs and heads ($p<0.042$, Mann-Whitney test) from *Ae. aegypti* (Bora). Similarly, significant differences in mean CCI were

obtained for *An. coluzzii* (Dkr), between thoraxes and legs (*p<0.0001*, Mann-Whitney test), thoraxes and heads (*p<0.0001*, Mann-Whitney test), and legs and heads (*p<0.009*, Mann-Whitney test). These results underlined a decrease in MS spectra reproducibility from thoraxes to legs and heads. Interestingly, the low mean CCI-values obtained for pairwise comparisons of MS spectra from two distinct body part for both species, ranging from 0.23 ± 0.06 (mean ± SD) to 0.47 ± 0.10, confirmed that these MS profiles are body part specific (Figure 4).

Efficiency of mosquito identification according to body part and homogenization modes by MS

The MS spectra used for MSP dendrogram analysis were included as reference MS spectra to create the DB1 (Table 1, Additional file 2). Then, each body part (legs, thoraxes and heads) from eight specimens per species (*Ae. aegypti* (Bora) and *An. coluzzii* (Dkr)) homogenized by MP, PP or TL, corresponding to a total of 144 samples, were submitted, in quadruplicate, to MALDI-TOF MS and queried against DB1 (Figure 1A). All the samples were correctly classified at the species and body part levels (Figures 5A and 5B). Except for the MS spectra from three *An. coluzzii* (Dkr) head samples, highly relevant identification scores were obtained (LSVs ≥ 2.0), independently of the homogenization mode used. The elevate LSVs indicated the high quality and reproducibility of MS spectra.

To assess the performances of MALDI-TOF MS for mosquito identification according to homogenization mode, LSVs were compared for each body part and species (Figure 5A-B). No significant difference (*p>0.05*, Kruskal Wallis test) was noticed between the homogenization modes per body part, except for legs from *An. coluzzii* (Dkr) (*p=0.02*, Kruskal Wallis test, Figure 5B). Although LSVs from the legs of *An. coluzzii* (Dkr) obtained with the automatic mode (TL) were significantly lower than those from MP (*p=0.01*, Mann Whitney test), identification scores remained highly relevant (LSVs>2.2), preventing misidentification risk. LSVs from legs of *An. coluzzii* (Dkr) were not different between homogenisation modes.

Interestingly, the comparison of the LSVs per homogenization mode, independently of the body part, revealed no significant differences (*p>0.05,* Kruskal Wallis test) for both species (Figure S2A-B). Conversely, significant different LSVs were obtained among the body part for *Ae. aegypti* (Bora) (*p<0.001* Kruskal Wallis test) and *An. coluzzii* (Dkr) (*p=0.028*, Kruskal Wallis test), independently of the homogenization mode used ((Figure S2C-D). Paired comparisons revealed a significant better matching against DB1 of MS spectra from thoraxes compared to legs (*p<0.01*, Mann Whitney test) or to heads (*p<0.001*, Mann Whitney test) from *Ae. aegypti* (Bora). For *An. coluzzii* (Dkr), LSVs from thoraxes were also significantly higher than heads (*p=0.023*, Mann Whitney test). These results indicated that the higher LSVs were obtained with MS spectra from thoraxes followed by legs and heads, confirming the data obtained on MSP dendrogram or CCI analyses.

Duration of sample processing according to body part and homogenization mode

To determine which homogenization method is the more advantageous, the time required for samples processing per homogenization mode was measured and estimated for larger specimen collection as complementary criteria. Then, heads, legs and thoraxes from five *Ae. aegypti* (Bora) were ground with MP, PP or TL by two experimenters and processing duration were recorded. For both manual modes, sample homogenizations were quicker for heads than for legs, than for thoraxes (Supplementary Table S1). Among the manual grinding modes, the PP method was more than 1.5 less time consuming than MP. However, except when the number of samples to process was very low (i.e., less than five), the automatic sample homogenization mode with TL was largely more rapid than both manual methods, independently of the body part. TL apparatus allows processing from 1 to 48 samples in only 3 minutes, whereas 1 to 36 or until 58 minutes were estimated to grind the same quantity of samples with PP or MP, respectively (Figure 5C). TL was then the faster method for sample homogenization, independently of the manipulator, the number of samples to process, or the body part selected. The automatic procedure seemed to be the more appropriate method for samples homogenization and was used for the successive experiments.

Consequence of mosquito blood meal on MS profiles according to body parts

To assess whether mosquito blood feeding status could affect MS profiles and subsequent mosquito identification, heads, thoraxes and legs from *Ae. aegypti* (Bora) and *An. coluzzii* (Dkr) collected kinetically 2, 6, 12, 24, 48 and 72 hours post-engorgement were analysed by MALDI-TOF MS (Figure 1B). MS spectra from heads, thoraxes and legs of not engorged *Ae. aegypti* (Bora) and *An. coluzzii* (Dkr) specimens, as well as MS spectra from human blood provided for mosquito meals, were used as the control for MS profile comparisons (Figure S3).

MS spectra of high intensity were obtained for the twenty specimens per species and body part tested at each time point. At 72 hours post-blood feeding, only 10 specimens were tested. The visual comparison of the 660 MS spectra using Flex Analysis v3.4 revealed that, for the vast majority of the samples (upper than 80% of the samples), no apparent change was noticed compared to respective body part and species from unfed specimens (Table S2). In the samples in which MS profile changes were observed, these modifications corresponded to the apparition of MS peaks at about 7,568 m/z and 15,138m/z (Figure S3). These two MS peaks, also present in MS profiles from human blood, were considered as blood contaminants of the mosquito MS spectra. These foreign MS peaks were found in all body parts and 2 to 48 hours post-feeding. Interestingly, the intensity of peaks corresponding to human blood signature decreased with the increasing delay post-blood feeding (Figure S3). This observation could likely be attributed to the digestion process of blood meal. However, this blood signature was more frequently found in the thorax samples (Table S2).

Identification of engorged mosquitoes by MS

To assess the consequences of blood engorgement on the identification of mosquitoes, MS spectra from the 660 samples were queried against DB1 (Figure 1B). The proportion of correct and relevant (LSVs ≥ 1.8) identification reached 96.5% (n=637/660) for MS spectra from both species independently of the body part and delay post-feeding (Figure 6). Among the 23 samples identified without relevant LSVs (i.e., <1.8), 11 belonged to thoraxes of *Ae. aegypti* (Bora), and 12 from *An. coluzzii* (Dkr) distributed in heads (n=1), legs (n=3) and thoraxes (n=8). The detection of MS peaks from blood origin was visible in half of them (n=12/23), all from thorax.

The comparison of LSVs between MS spectra with and without blood foreign peaks for each mosquito species revealed a significant decrease in matching scores ($p<0.001$, Mann-Whitney test) only for MS spectra from *Ae. aegypti* (Bora) (Figure S4A-B). Nevertheless, the proportion of correct and relevant (LSVs ≥ 1.8) identification for mosquito MS spectra with or without blood foreign peaks remained high, reaching 90.6% (n=116/128) and 97.9% (n=521/532), respectively. Regarding mosquito body parts, MS spectra from thoraxes obtained significant lower LSVs for *Ae. aegypti* (Bora) ($p<0.001$, Mann-Whitney test, Figure S4C) and *An. coluzzii* (Dkr) ($p<0.004$, Mann-Whitney test, Figure S4D). Although blood foreign MS peaks seem to affect more thoraxes match scoring, correct and relevant LSVs (>1.8) were obtained for the large majority of thoraxes spectra with human blood MS peaks (80.3%, n=49/61).

Impact of mosquito origin on the identification and LSV distribution

To assess whether MS spectra variations occurred for specimens from the same species but from distinct geographical origins, MS spectra from 4 distinct mosquito species, laboratory-reared or field collected, were queried against DB2 and DB3. The median LSVs against DB2 were 2.21, 2.32 and 2.36 for heads, legs and thoraxes, respectively, irrespective of the species analysed. The distribution of LSVs varied significantly between body parts (p=0.002, Kruskal Wallis test), with the heads obtaining the lowest scores (Figure S5). The proportion of correct and relevant (LSVs>1.8) identification against DB2 ranged from 79.0% for heads to 83.9% for legs (Table 2). The query of these MS spectra against the DB3, upgraded with MS spectra from field specimens, did not improve significantly ($p>0.05$, Chi-square tests), the proportion of correct and relevant identification. Conversely, LSVs obtained per body part per field species were significantly improved between DB2 and DB3 for nearly all paired comparisons ($p<0.05$, Mann-Whitney test, Figure 7). Interestingly,

misidentification concerned mainly MS spectra from *An. gambiae* s.l., underlining the difficulty to classify specimens from species complex.

Discussion

The MALDI-TOF MS profiling has revolutionized clinical microbiology to identify microorganisms [45,46], and its versatility, robustness, and practice allowed its application to modernize arthropod monitoring for a decade [15]. In addition to its success for the identification of specimens from more than a dozen of arthropod families, including Culicidae [15, 47], this innovative proteomic tool was pragmatically used for the detection of pathogenic agents in vectors [48–50] or the determination of their trophic preferences [42, 51]. Currently, the main limitation to its wide usage is the absence of a public reference MS spectra database with arthropod spectra formally certified after reliable morphological and molecular identification. Nevertheless, prior to creating and sharing reference MS spectra database, establishing a standardised protocol becomes compulsory. The absence of consensus procedures for sample preparation contributed to results heterogeneity, hampering comparison and sharing of MS spectra [24, 44, 49]. Then, the present study assessed some intrinsic parameters, such as body part selected from the mosquito for MS submission or geographical origin, but also extrinsic factors, like the blood-feeding status or the mode of sample homogenization, which are factors changing moderately to markedly the resulting MS spectra [24, 44, 49].

Although mosquito legs were the body part the most frequently used for specimen identification by MS analysis [26, 27, 30, 31], for which a standardized protocol was established [29][44], other anatomic parts were selected, including thoraxes [32, 33], cephalothoraxes [34, 35] or heads [24]. These last body parts were chosen, notably, to prevent the risk of non-identification from specimens having lost all their breakable legs. Here, the analysis of MS spectra between three body parts from two mosquito species using CCI revealed that the higher reproducibility of protein profiles was obtained for thoraxes followed by legs and heads. The lower branch distances obtained by cluster analysis for legs and thoraxes compared to heads for both species underlined a more important heterogeneity of MS profiles for this last body part. Moreover, the significant higher LSVs obtained for thoraxes compared to legs and heads confirmed that thoraxes appeared as the most appropriate body part for mosquito identification by MS profiling. Nevertheless, the high proportions (90%) of correct and relevant identification (LSVs > 1.8) obtained for legs and heads supported that both body parts could also be used.

Conversely to our findings, a recent work pointed out that mosquito heads generated MS spectra with the highest reproducibility compared to legs and thoraxes [24]. These inconsistent results highlighted the necessity to propose guidelines for mosquito species identification by MS profiling. The divergences could be attributed to numerous factors that impact the quality of MS spectra, such as the storing conditions of field-collected specimens and the protocol applied [29, 36]. In the Nabet et al study, the sample homogenization was performed using a unique volume of mix buffer independently of the body part [24], whereas, here, the most appropriate volume of mix buffer was selected based on our experiments for heads or previous demonstration done on legs and thoraxes [28, 32]. Using an inadequate volume of mix buffer could reduce protein extraction and could likely explain in part the heterogeneity of MS profiles per body part [37].

The homogenization mode of samples was also reported to impact the quality of MS spectra [29]. Here, no significant differences in LSVs were noticed between the two manuals and the automatic methods, independently of the body part and species tested. The high reproducibility obtained with both manual modes was likely attributed to the low number of samples treated by experimented and meticulous manipulators. The performances of manual grindings are probably inversely proportional to the number of samples to process and directly linked to the skills of the experimenters. Moreover, the manual grinding became a bottleneck in the pipeline of mosquito identification due to the time-consuming required by this step. Effectively, the estimated time required to process 24 samples was, respectively, six to ten-fold

longer using PP and MP compared to TL mode. As MALDI-TOF MS is well-adapted for high throughput analyses, applying an automatic homogenization procedure limiting handling variations and allowing for testing a large number of samples appears more appropriate. The establishment of an automatized procedure for mosquito larvae homogenisation [29, 37] allowed the application of MALDI-TOF MS to monitor mosquito fauna at immature stages for several months [52, 53]. The homogenization step was automatized to reduce improper sample grinding and improve the acquisition of high-quality MS spectra. Currently, no quality control step to reject outlier MS spectra is available on commercial software (e.g., MALDI-Biotyper v3.0. from Bruker Daltonics). Some R packages were developed to examine MS spectra quality [54, 55], but they require a minimum of computational knowledge. In the future, the systematic application of a quality control step of MS spectra prior to their query against MS reference DB will allow for filtering of atypical spectra and then prevent inaccurate identification.

To improve mosquito identification, notably for sibling or cryptic species, it was proposed to submit to MS, independently, two distinct body parts from the same specimen. This strategy applied to the thoraxes and legs of mosquitoes allowed to corroborate the identification obtained per body part and enhanced the identification confidence level [32, 33]. The same strategy applied to ticks permitted the classification of unambiguously closely related *Ixodes* species [21]. The advantages of testing more than one body part are that in case of a damaged specimen, at least one of the body parts selected remained intact for MS submission. For cryptic species, the double DB query with distinct compartments could confirm identification in case of doubt. Interestingly, herein, cluster analysis showed that thorax and head MS spectra were on closer branches to each other than against the legs for both species. This vicinity reflects the proximity of the thorax and head MS profiles. The risk of cross-matching of MS spectra between thoraxes and heads of the same species was more probable and was already reported [24]. Then, in the case of the use of two body parts from the same specimen to improve and corroborate mosquito species identification, it appears more judicious to pair legs and thoraxes or legs and heads rather than the couple heads and thoraxes.

The impairment of arthropod identification by MS from freshly blood-engorged specimens was already reported [19, 56]. In sandflies, this failure of specimen identification was attributed to the potential presence of blood traces in the thoraxes impacting MS patterns by masking species-specific biomarker masses [56]. Conversely, others, assessing the performances of MALDI-TOF MS for determining host blood origin from engorged sand flies using abdomens, successfully identified these field-collected specimens by submitting respective thoraxes to MS [57]. Here, no drastic MS profile change was noticed for engorged mosquitoes from both species independently of the body part and delay post-feeding compared to unfed specimens. More than 95% of the samples were correctly and relevantly identified. Blood signature was detected in only 19.4% of the samples tested from engorged specimens, represented by mainly two MS peaks, corresponding presumably to the mono- (15,138 m/z) and double-charge (7,568 m/z) of the same kind of protein. This 15 kDa MS peak was already observed in engorged sand flies [58] and mosquitoes [42].

The inconstant detection of the blood MS peaks in engorged specimens, the detection of the same blood MS peaks in MS spectra from the three body parts, plus the inverse correlation of blood peak intensity with the delay of blood-feeding support the hypothesis that the origin of blood signatures come likely from contamination which occurred during dissection of engorged specimens rather than the remaining of host blood. Effectively, during the separation of the thorax from the abdomen of freshly engorged specimens, blood leakage could occur, compromising the cleanness of the other body parts, as reported in other arthropods [57, 58]. Nevertheless, as the amount of blood that stained the other body parts is generally low, MS specimen identification remains generally possible [27, 58]. One work reported a decrease in the identification rate of MS from the thoraxes of blood-fed mosquitoes [24]. In the same study, MS spectra from thoraxes of unfed mosquitoes were also less efficient for specimen identification compared to legs or heads. It is then likely that other factors, such as the sample preparation protocol, could explain the lower reproducibility of mosquito thorax MS spectra [24]. To limit blood contamination, the engorged specimens could be frozen and dissected onto a refrigerated plate to prevent sample thawing and, subsequently, blood leak.

The description of MS profile variations according to the geographical origin was already reported for mosquitoes at the adult [24, 35, 38] or immature stages [37], but also other families, such as sand flies [59] or ticks [21, 22, 60]. However, these MS spectra variations generally did not hamper the correct identification. Here, the addition of MS spectra from field specimens did not significantly improve the proportion of correct and relevant identification for the three body parts. Nevertheless, the DB upgrading significantly increased the LSVs from field specimens. The present work confirmed that some variations of MS spectra occurred according to geographical origin for specimens from the same species, which were observed for the three body parts. However, others factors such as the storing conditions [29] or duration of storing [44] could also participate in intra-species MS profile variations. Then, MS spectra from specimens from the same species collected in different areas remain sufficiently close for their correct and relevant identification despite the absence of MS spectra of specimens from all locations prospected included in the DB. Nevertheless, as these MS spectra improve the score of identification confidence, the introduction of MS spectra from local specimens is recommended.

Conclusions

The interest in MALDI-TOF MS profiling was notably attributed to its advantages over molecular methods and morphological identification, offering reagent low-costs, fast and straightforward sample preparation and data analyses, which does not require specialized expertise. As several factors can affect MS spectra and, consequently, species classification, the proposition of procedures for new users hoping to apply the MALDI-TOF MS tool for mosquito identification become unavoidable. The homogenization of samples using automatic systems appeared more appropriate, notably for a large number of samples, standardizing the grinding parameters and reducing the processing time. Heads, legs and thoraxes were effective for mosquito identification; however, the higher reproducibility of MS profiles from thoraxes followed by legs and finally heads evidenced the distinct level of performance according to the body part. Moreover, the high diversity of legs and thorax MS spectra from the same specimen could improve species identification rate and level of confidence by independent MS submission. Blood signatures could be detected in MS spectra from heads, legs and thoraxes of engorged mosquitoes, with an infrequent impairing of mosquito identification. As geographical origin induces heterogeneity of MS spectra from specimens of the same species, implementing reference DB with MS spectra from region-specific specimens circumvented this limitation. Finally, the present work established guidelines concerning the selection of mosquito anatomic parts and the modality of sample preparation for future specimen identification by MALDI-TOF MS profiling. These protocols could be used as references for creating an international MS database.

Abbreviations

a.u.
arbitrary units
CCI
Composite Correlation Index
CI
confident interval
PCR
Polymerase Chain Reaction
LSVs, log score values
MALDI-TOF MS
Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry
MP

micropipette
min.
minute
m/z
mass-to-charge ratio
PP
pellet pestle
Sec.
second
sl
sensu lato
ss
sensu stricto
TL
TissueLyser.

Declarations

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Availability of data and materials

The datasets of MS reference spectra added to the MS DB in the current study are freely available and downloadable from the Additional file 2.

Authors' contributions

Conceived and designed the experiments: LA, MMC, RB. Performed the experiments: MMC, RB. Analyzed the data: RB, MMC, LA. Contributed reagents/materials: AZD, AJM, PP. Drafted the paper: RB, MMC, LA. Revised critically the paper: all the authors.

Ethics approval and consent to participate

The study was conducted under the ethical clearance N° 2018/06/1036/CE/CNERSH/SP and N° 1284/CRERSHC/2021 granted by the Cameroon National (CNE) and Centre Regional (CRE) Ethics Committee for Research on Human Health. Authorization to carry out the study was obtained from administration and heads of household (HoH) through of an inform consent form.

Consent for publication

Not applicable.

Conflict of interests

The authors declare that they have no competing interests.

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Tables

Table 1. Overview of mosquito origins, subgroups for DB creation and parameters assessed.

Species	Strains[a]	Country	Source	Stored	N (engorged)[b]	DB1[c]	DB2[c]	DB3[c]	Parameters assessed[d]
Ae. aegypti	Bora Bora (Bora)	French Polynesia	Lab.	Fresh	155 (110)	6*	6*	6*	A, B, C
Ae. aegypti	Oiapoque (Oia)	Brazil	Field	Frozen (-20°C)	15			2#	C
Ae. albopictus	Marseille (Mrs)	France	Lab.	Fresh	15		2#	2#	C
Ae. albopictus	Dschang (Dsg)	Cameroon	Field	Frozen (-20°C)	15			2#	C
An. coluzzii	Dakar (Dkr)	Senegal	Lab.	Fresh	155 (110)	6*	6*	6*	A, B, C
An. gambie s.s.	Kisumu (Kis)	Kenya	Lab.	Frozen (-20°C)	20		2#	2#	C
An. coluzzii	Tibati (Tib)	Cameroon	Field	Silicate, RT	16			2#	C
An. gambie s.s.	Brazaville (Bra)	Congo	Field	Silicate, RT	17			2#	C
Total					**408 (120)**	**12**	**16**	**24**	

[a]Abbreviate name of each strain is indicated into brackets.

N, Number of specimens; [b]Number of engorged specimens is indicated into brackets.

[c]Number of specimens included in the DBs per body part and homogenization mode.

[d]Classification of parameters assessed according to Figure 1, A: Effects of homogenization mode and body parts; B: Effect of blood meal; C: Effect of geographical origin.

*2 specimens per body part homogenized either by MP, PP or TL mode.

[#]2 specimens per body part homogenized by TL mode.

DB, database; MP, micropipette; PP, pellet pestle; TL, TissueLyser; RT, room temperature.

Table 2. MALDI-TOF MS identification results of species from different origins after crosswise testing against DB2 and DB3.

Query against				Correct species, n (%)			Incorrect species, n (%)		
Databases[§]	**Species**	**Origin[a]**	**N[b]**	**Heads**	**Thoraxes**	**Legs**	**Heads**	**Thoraxes**	**Legs**
BD2	*Ae. aegypti**	French Polynesia (Bora)	15	15 (100)	15 (100)	15 (100)	0 (0.0)	0 (0.0)	0 (0.0)
	Ae. aegypti	Brazil (Oia)	15	15 (100)	15 (100)	15 (100)	0 (0.0)	0 (0.0)	0 (0.0)
	*Ae. albopictus**	France (Mrs)	13	13 (100)	13 (100)	13 (100)	0 (0.0)	0 (0.0)	0 (0.0)
	Ae. albopictus	Cameroon (Dsg)	15	15 (100)	15 (100)	13 (86.7)	0 (0.0)	0 (0.0)	2 (13.3)
	*An. coluzzi**	Senegal (Dkr)	15	5 (33.3)	12 (80.0)	15 (100)	10 (66.7)	3 (20.0)	0 (0.0)
	*An. gambiae s.s.**	Kenya (Kis)	18	18 (100)	18 (100)	18 (100)	0 (0.0)	0 (0.0)	0 (0.0)
	An. coluzzii	Cameroon (Tib)	16	4 (25.0)	0 (0.0)	4 (25.0)	12 (75.0)	16 (100)	12 (75.0)
	An. coluzzii	Congo (Bra)	3	2 (66.7)	0 (0.0)	0 (0.0)	1 (33.3)	3 (100)	3 (100)
	An. gambiae s.s.	Congo (Bra)	14	11 (78.6)	14 (100)	11 (78.6)	3 (21.4)	0 (0)	3 (21.4)
Total (%)			**124**	**98 (79.0)**	**102 (82.3)**	**104 (83.9)**	**26 (21.0)**	**22 (17.7)**	**20 (14.5)**
BD3	*Ae. aegypti**	French Polynesia (Bora)	15	15 (100)	15 (100)	15 (100)	0 (0.0)	0 (0.0)	0 (0.0)
	Ae. aegypti	Brazil (Oia)	13	13 (100)	13 (100)	13 (100)	0 (0.0)	0 (0.0)	0 (0.0)
	*Ae. albopictus**	France (Mrs)	13	13 (100)	13 (100)	13 (100)	0 (0.0)	0 (0.0)	0 (0.0)
	Ae. albopictus	Cameroon (Dsg)	13	13 (100)	13 (100)	13 (100)	0 (0.0)	0 (0.0)	0 (0.0)
	*An. coluzzi**	Senegal (Dkr)	15	5 (33.3)	13 (86.7)	15 (100)	10 (66.7)	2 (13.3)	0 (0.0)
	*An. gambiae s.s.**	Kenya (Kis)	18	18 (100)	18 (100)	18 (100)	0 (0.0)	0 (0.0)	0 (0.0)
	An. coluzzii	Cameroon (Tib)	14	10 (71.4)	6 (42.9)	5 (35.7)	4 (28.6)	8 (57.1)	9 (64.3)
	An. coluzzii	Congo (Bra)	3	2 (66.7)	0 (0.0)	1 (33.3)	1 (33.3)	3 (100)	2 (66.7)
	An. gambiae	Congo (Bra)	12	4 (25.0)	11 (91.7)	5 (41.66)	8 (75.0)	1 (8.3)	7 (58.3)

s.s.							
Total (%)	**116**	**93 (80.2)**	**102 (87.9)**	**98 (84.5)**	**23 (19.8)**	**14 (12.1)**	**18 (15.5)**

[a]Country of origin of the sample with abbreviate name of each strain indicated into brackets.

[b]Number of sample blindly tested against databases (DB2 or DB3).

[§]DB2 and DB3 composition are available in Table 1.

n (%): number of sample with correct (same name with molecular identification) or incorrect (different name with molecular identification) species identification, %, prevalence or proportion of samples

*: indicate mosquito strains laboratory reared.

DB: database.

Figures

Figure 1

Experimental design of study. The different parameters evaluated were (**a**) the homogenization procedures per body part, (**b**) the consequences of engorgement per body part and (**c**) the effect of mosquito origin on mosquito identification per body part. The mosquito species, their geographical origin and the number of specimens tested were indicated. The homogenization mode used was indicated in the diamond form. See the table 1 for details about composition of DB1, DB2 and DB3 and mosquito species origins. MP, micropipette; PP, pellet pestle; TL, TissueLyser; MS DB, Mass spectrometry database.

Figure 2

Comparison of MALDI-TOF MS spectra from heads, legs and thoraxes of *Aedes aegypti* (Bora) and *Anopheles coluzzii* (Dkr) homogenized using distinct methods. Representative MS spectra from heads (red), legs (green) and thoraxes (blue) of (**a**) *Ae. aegypti* (Bora) and (**b**) *An. coluzzii* (Dkr). MS spectra from two distinct specimens per body part and homogenization method were presented using FlexAnalysis v3.4 software. MP, micropipette; PP, pellet pestle; TL, TissueLyser; a.u., arbitrary units; m/z, mass-to-charge ratio.

Figure 3

MSP dendrogram of MALDI-TOF MS spectra from heads (red), legs (green) and thoraxes (blue) of *Aedes aegypti* (Bora) and *Anopheles coluzzii* (Dkr) homogenized using distinct methods. Two specimens per species, per body part and per homogenization method are presented. The distance units correspond to the relative similarity of MS spectra. The dendrogram was created by Biotyper v3.0 software. MP, micropipette; PP, pellet pestle; TL, TissueLyser.

Figure 4

Composite Correlation Index (CCI) matrix value representing the levels of MS spectra reproducibility between head, legs and thoraxes of (a) *Aedes aegypti* (Bora) and (b) *Anopheles coluzzii* (Dkr) homogenized with distinct modes. The levels of MS spectra reproducibility are indicated in red and blue, revealing relatedness and incongruence between spectra, respectively. The values correspond to the mean coefficient correlation and respective standard deviations obtained for paired condition comparisons. Bold numbers correspond to CCI values obtained for each body part independently of the homogenization mode used. CCI was calculated with MALDI-Biotyper v3.0 software. MP, micropipette; PP, pellet pestle; TL, TissueLyser.

Figure 5

Evaluation of performances from each homogenization mode taking into account sample identification accuracy and duration of sample preparation. LSVs from MS spectra of heads, legs and thoraxes of (**a**) *Ae. aegypti* (Bora) and (**b**) *An. coluzzii* (Dkr), using distinct homogenization methods were compared. Dashed line represents the threshold values (LSV ≥ 1.8), for relevant identification. Significant differences between homogenization modes per body part were indicated (Kruskal Wallis test). The duration of sample preparation according to homogenization methods was presented (**c**). This graphic was performed based on average data measured and estimated using legs, heads and thoraxes from five mosquito samples homogenized either with MP (orange), PP (green) or TL (blue) modes (detail in Table S1). The time required for processing 24 samples according to homogenization mode are presented. a.u., arbitrary units; LSV, log score value; min, minutes, ns, not significant; MP, micropipette; PP, pellet pestle; TL, TissueLyser.

Figure 6

Consequences of blood engorgement on mosquito identification per body part.

LSVs from MS spectra of head, legs and thorax of (**a**) *Ae. aegypti* (Bora) and (**b**) *An. coluzzii* (Dkr), kinetically collected after human blood engorgement were compared. Collection time points were distinguished by color code as indicated in the right panel. Dashed line represents the threshold values (LSV ≥ 1.8), for relevant identification. All samples were homogenized using TL mode. a.u., arbitrary units; LSV, log score value; h, hours; TL, TissueLyser.

Figure 7

Effect of geographical origin on mosquito identification per body part. LSVs from MS spectra of field collected mosquito species (**a**) *An. gambiae s.s.* (Bra), (**b**) *An. coluzzii* (Tib), (**c**) *Ae. albopictus* (Dsg) and (**d**) *Ae. aegypti* (Oia) against DB2 (pink) and DB3 (blue). Significant differences of LSVs obtained between DB2 and DB3 per species and body part were indicated (Mann-Whitney test). Dashed line represents the threshold values (LSV ≥ 1.8), for relevant identification. All samples were homogenized using TL mode. a.u., arbitrary units; LSV, log score value; TL, TissueLyser.

Supplementary Files

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- FigureS1.pdf
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