Annales Africaines de Médecine
Article original

Contribution of MALDI-TOF to the identification of Anopheles gambiae complexes in Kinshasa, Democratic Republic of Congo

Contribution de MALDI-TOF à l'identification du complexe Anophèles gambiae à Kinshasa, République démocratique du Congo

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Résumé

Contexte & objectif. Anopheles gambiae s.l. reste la principale espèce vectrice responsable de la transmission du paludisme en RDC. L'identification précise et rapide de cette espèce est cruciale pour adapter les interventions de lutte antivectorielle. La spectrométrie de masse par désorption-ionisation laser assistée par matrice (MALDI-TOF MS) est apparue comme une technique prometteuse pour l'identification des vecteurs de moustiques en raison de sa précision et de sa reproductibilité. *Méthodes*. Cette étude transversale et analytique a été réalisée à Kinshasa, où des larves d'*Anopheles* ont été collectées entre septembre 2021 et mai 2022. Après élevage, les spécimens adultes ont été identifiés morphologiquement et soumis à une identification moléculaire par PCR pour différencier les espèces du complexe *Anopheles gambiae*. Les échantillons ont ensuite été analysés par MALDI-TOF MS, en utilisant les profils protéiques de chaque partie anatomique pour créer une base de données de référence des espèces locales. *Résultats.* L'analyse moléculaire a montré une forte présence d'*An. gambiae* (93%). L'analyse par MALDI-TOF MS a trouvé une haute reproductibilité des profils spectraux, avec un score logarithmique supérieur à 2 pour 72 % des échantillons. Les têtes ont fourni les meilleurs résultats en termes de qualité spectrale. La validation croisée a montré que les têtes étaient les plus fiables pour l'identification des espèces, avec un score de reconnaissance de 95,43 %. *Conclusion.* La technique MALDI-TOF MS s'est révélée

Summary

Context and objective. Anopheles gambiae s.l remains the main vector species responsible for malaria transmission in the Democratic Republic of Congo (DRC). The precise and rapid identification of this species is crucial for adapting vector control interventions. Matrix-assisted laser desorption-ionisation -Time of Flight mass spectrometry (MALDI-TOF MS) has emerged as a promising technique for the identification of mosquito vectors because of its accuracy and reproducibility. *Methods*. This analytical cross-sectional study was carried out in Kinshasa, where *Anopheles* larvae were collected between September 2021 and May 2022. After rearing, adult specimens were morphologically identified, followed by PCR techniques to differentiate species of the *Anopheles gambiae* complex. The samples were then analysed by MALDI-TOF MS, using protein profiles for each anatomical part to create a reference database for local species. *Results.* Molecular analysis revealed a high presence of *An. gambiae* (93%). MALDI-TOF MS analysis showed high reproducibility of spectral profiles, with a log score greater than 2 for 72% of samples. Insect heads provided the best results in terms of spectral quality. Cross-validation showed that heads were the most reliable samples for species identification, with a recognition score of 95.43%. *Conclusion.* The MALDI-TOF MS technique proved to be effective for the identification of *Anopheles* mosquitoes in Kinshasa, with good spectral reproducibility and accurate species identification.

efficace pour l'identification des moustiques *Anopheles* à Kinshasa, avec une bonne reproductibilité spectrale et une identification précise des espèces.

Mots-clés : *Anopheles gambiae* s.l, Identification, Maldi-Tof, Kinshasa
Reçu le 16 octobre 2024
Accepté le 29 mai 2025
https://dx.doi.org/10.4314/aamed.v19i1.2

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Keywords: *Anopheles gambiae* s.l, Identification, MALDI-TOF, Kinshasa

Received: October 16th, 2024
Accepted: May 29th, 2025
https://dx.doi.org/10.4314/aamed.v19i1.2

Introduction

Vector-borne diseases such as malaria pose significant public health challenges globally, and especially in sub-Saharan Africa. It is still rife in a number of countries around the world, with varying degrees of morbidity (1-2). The World Health Organization (WHO) estimates that the occurrence of malaria remains exceedingly high in sub-Saharan Africa, which bears nearly 95% of the global malaria burden (2). This high prevalence is largely due to climatic conditions that favour the proliferation of specific vector species, in particular those that efficiently transmit *Plasmodium falciparum*, the most virulent malaria parasite (3). The situation is further exacerbated by inadequate healthcare infrastructure, difficult socio-economic conditions, low levels of awareness and population mobility (3-4). The Democratic Republic of Congo (DRC), located in central Africa, is one of the most severely impacted countries with an exceptionally high incidence of malaria (5). The National Malaria Control Programme in the DRC focuses on controlling both the parasites and the vectors. Since 2015, vector control measures such as the distribution of insecticide-treated nets and indoor residual spraying, have significantly reduced malaria incidence and mortality in sub-Saharan Africa (3,6). To sustain these achievements and advance towards the elimination of malaria, it is crucial to implement vector-specific control measures tailored to the local vector species. Monitoring changes in vector species composition and behaviour allows for on-going adjustments in control programmes to enhance their effectiveness (7). Targeted interventions directed at the predominant vectors in specific regions can significantly decrease malaria transmission (8). Detailed and precise identification of vectors is essential for

understanding the dynamics of malaria transmission (9). By exploring seasonal and geographical variations in vector species, high-risk periods and areas can be identified, facilitating the planning of targeted interventions during peak transmission periods in the most affected areas (9). Traditional methods for identifying malaria vectors, including morphological analysis and molecular techniques, have limitations and this highlights the need for more efficient alternatives. Morphological techniques require meticulous inspection under a microscope, which is laborious and time-consuming (10). Molecular techniques, such as DNA sequencing or PCR, involve complex sample preparation, use of specific reagents and expensive equipment, and are also time-consuming (11). Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) represents an emerging technique that offers several advantages over traditional methods (12). It permits rapid and accurate identification of mosquitoes by generating unique protein profiles for each species. It reduces identification errors due to morphological variations and makes it possible to differentiate between closely related species (12). Additionally, MALDI-TOF MS results are highly reproducible, which is crucial for epidemiological studies and long-term surveillance programmes (13). The use of MALDI-TOF for the identification of *Anopheles* species in Africa is not widespread in Africa, whereas its effectiveness is proved in other regions. Establishing a reference database of species specific to Kinshasa is vital for supporting future research and improving entomological surveillance. This database would not only help users understand vector distribution in a city with heterogeneous ecological characteristics but also strengthen local diagnostic and surveillance capabilities. It could also serve as a model for other regions facing similar challenges (14). Therefore, the aim of the present study was to evaluate the potential of MALDI-TOF MS for identifying *Anopheles* species collected in Kinshasa, and to contribute to the creation of a local reference database.

Methods

Study sites

The present research was conducted in the city-Province of Kinshasa, located in the southwest of the DRC (4°19' 30" S and 15°19' 20" E). Kinshasa is a megapolis covering an area of 9965 km² and with an estimated population of 17 million inhabitants (15). It consists of 24 communes divided into four districts (Tshangu, Mont Amba, Lukunga, and Funa). Kinshasa experiences a tropical hot and humid climate, characterized by two distinct seasons: a dry season and a rainy season (16). The expansion of market gardening activities, the absence of rainwater drainage systems, and poor waste management all contribute to the pollution of surface waters. The long rainy season lasts about nine months and facilitates the formation of numerous temporary larval habitats, such as puddles and marshes, which are ideal breeding grounds for *Anopheles* larvae (17). This study was conducted across all four districts of Kinshasa. Sampling points were chosen based on the size of the district and the accessibility of larval breeding sites (Figure 1).

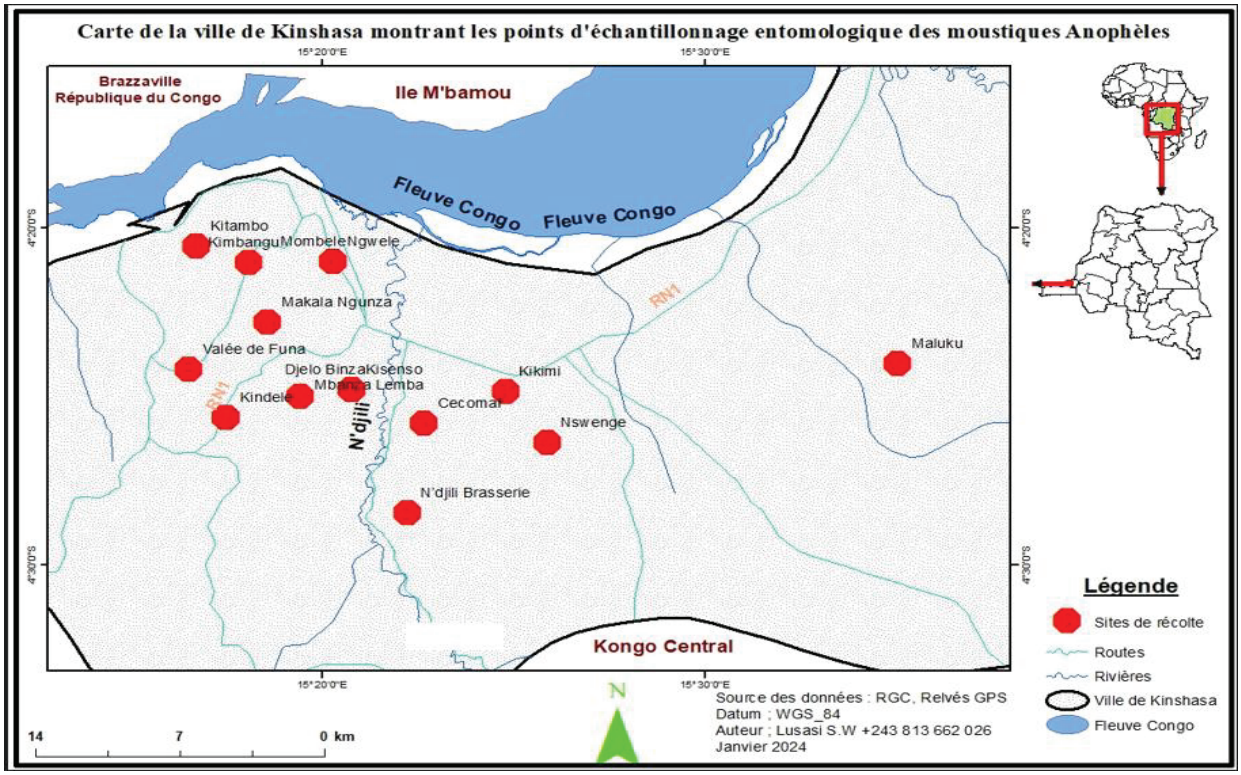

Figure 1. Maps of the city of Kinshasa showing the sampling sites of *Anopheles*

Study design and sampling procedure

This study was designed to evaluate the performance of MALDI-TOF MS for identifying malaria vectors within the *Anopheles gambiae* complex in Kinshasa, DRC. Larvae were collected across four districts and reared into adults. Morphological and PCR-based molecular identification confirmed species identity. Specimens were dissected, and protein profiles of anatomical parts were analyzed using MALDI-TOF MS. A local spectral reference database was built using high-quality spectra (log score >2), and identification accuracy was validated with MSI-2 software. This integrative approach enabled reliable and reproducible identification of *Anopheles* species in a region-specific context.

Several larval collections were conducted over a period from September 2022 to May 2023. In each district, at least one area with accessible potential breeding sites for *Anopheles* mosquitoes was selected. The larvae were collected using the "dipping" method (18). The genera of mosquito larvae (*Anopheles* or *Culex*) were identified based on their position relative to the water surface (19). To maximize the genetic diversity of *Anopheles* larvae, at least 200 larval habitats were selected along a 15 km transect in each study area. Only *Anopheles* larvae were retained and transported to the insectarium at the School of Public Health in Kinshasa for rearing into imagos (adults).

Anopheles mosquitoes aged 3 to 5 days, obtained after rearing, were morphologically identified under a binocular loupe using the taxonomic key proposed by Gillies and Coetzee (19). The identified *Anopheles* species, including *Anopheles gambiae* s.l. and *Anopheles funestus* gpe, were placed in Eppendorf tubes containing silica gel and then stored at -20°C and -40°C, respectively. At least 30 *An. gambiae* s.l. and all *An. funestus* gpe were selected across the four districts. These samples were sent to the Parasitology and Mycology Laboratory at the Pitié Salpêtrière Hospital (PSL) in Paris for preliminary molecular identification (PCR) before being subjected to proteomic approaches using MALDI-TOF.

DNA extraction and PCR amplification

The *Anopheles* specimens collected were initially dissected (head, abdomen, thorax/wings, and legs) using a binocular magnifying glass and entomological forceps. Only the abdomens were used for molecular analyses (gold standard) while the other anatomical parts were placed in 1.5 mL conical Eppendorf tubes for comparative MALDI-TOF analysis.

The abdomens were placed in stainless steel ball mill tubes containing eMAG® lysis buffer from bioMérieux, based on guanidine. The tubes were then incubated at room temperature overnight. DNA extraction was performed using the eMAG® automated extractor from bioMérieux, a technology based on magnetic silica extraction.

For the *Anopheles*, PCR amplification of the Intergenic Spacer (IGS) region allowed differentiation of phylogenetically close species within the *gambiae* complex, using a single nucleotide polymorphism (20). Two types of master mix were used: the mix *An. arabiensis / An. gambiae* to distinguish *An. arabiensis* from the "*An. gambiae/An. coluzzii*" group and the mix *An. gambiae / An. coluzzii* to distinguish *An. gambiae* and *An. coluzzii* with another primer system. The primers amplified fragments with theoretical sizes of 221 bp for *An. gambiae*, 333 bp for *An. coluzzii*, and 387 bp for *An. arabiensis* (20). Internal controls for each species being identified, sourced from reference centers, were used to verify band heights. The DNA extracts from *Anopheles funestus* gpe were stored for future sequencing analyses.

Protein extraction and mass spectra acquisition

The dissected heads, legs and thorax with wings were processed separately. They were transferred to individual 1.5 ml microcentrifuge tubes and rinsed in 70% ethanol for 10 minutes. Tubes were centrifuged at 13,000 rpm for 10 min, and the supernatant was then discarded. After a second centrifugation (13,000 rpm, 2 min), the remaining ethanol solution was then eliminated using a micropipette and left to evaporate. Protein extraction was performed after the addition of 10 µL of 70% formic acid. After manual homogenization with a micropipette, the homogenate was incubated for 5 min. Then, 10 ml of 100% acetonitrile was added and incubated for 5 min. The homogenate was centrifuged (13,000 rpm, 2 min), and 1 µL of the supernatant of each sample containing the protein extract was deposited onto a steel target plate (Bruker Daltonics, Wissembourg, France). Once dried, the deposits were covered with a 1-µL alpha-cyano- 4-hydroxycinnamic acid matrix prepared in 50% acetonitrile and 2.5% trifluoroacetic acid and 47.5% of HPLC grade water (final concentration of 10 mg/mL).

To ensure reproducibility, a total of ten replicates were spotted for each specimen to be included in the database, and a total of four replicates were spotted for each specimen of the panel to be tested, as previously published (21).

To obtain the mass spectra (characteristic protein fingerprint of an organism), the plate layout was initially configured using the MBT Compass software. The spectra were obtained after the default settings were applied with the Microflex LT software (Bruker France SAS). They were visualized using the AutoXecute option of the FlexControl v3.4 software (Bruker France SAS). The spectra were acquired in linear mode in positive ion mode at a laser frequency of 60 Hz and within a mass range of 2-20 kDa.

Construction of a mass spectra database

The construction of a database required establishing a reference spectrum (Main Spectrum Profile: MSP) for each specimen and each anatomical part. The MSP is the average spectrum calculated from the 4 technical replicates deposited on the plate. To ensure the best quality of our database, all poor-quality spectra were eliminated.

To do this, we verified the reproducibility of the results between the 4 deposits of the same specimen. We compared the average MSP spectrum with each of its deposits using a MALDI Biotyper v4.1 algorithm. Logarithmic score values (LSV) ranging from 0 to 3 were obtained. Unlike other studies that consider LSV observations between 1.7 and 1.8 as the threshold above which the results are interpretable (21,22), deposits resulting in LSV below 2 were considered non-reproducible and were removed from our database.

Panel Construction (Validation of the reference database)

To ensure the validation of our reference database, an analysis of spectra against the reference database was performed using MSI-2 software. This analysis allowed for the elimination of self-recognitions of individuals (the spectra of an individual's legs cannot be compared against the spectra of the head or thorax of the same individual). The identification of an *Anopheles* species was possible by querying the database with the MSI-2 software. Each of the 4 spectra from a given anatomical part was compared to the spectrum database. The reference spectra were created based on morphological and molecular analyses. The MSI-2 analysis also identified the most reproducible anatomical part.

Mass Spectra Analysis

To evaluate spectral variation across the entire database, a composite correlation index was calculated using the MALDI Biotyper v4.1 software with default parameters (mass range 3.0-12.0 kDa; resolution 4; eight intervals; autocorrection disabled). The correlation index matrix was represented as a heatmap. The correlation levels between the mass spectra are indicated from red to blue, which reveal the agreement and differences between the spectra respectively. Unsupervised clustering analysis (dendrogram) was performed on the basis of protein mass profiles (m/z, intensity) using MALDI Biotyper v4.1 software, to assess the relationships between mass spectra. The calculation mode was set to default parameters, distance was measured by correlation, linkage by average, and the score threshold for a single organism was 300 arbitrary units and 0 arbitrary units for related organisms. The proximity of an Anopheles spectrum to other spectra was reflected by an arbitrary distance level

Ethical Consideration

All research activities were carried out in accordance with the standards and codes of conduct accepted by the International Conference on Harmonisation (ICH).

Collection of larvae from identified larval collections within households required written permission from the heads of household.

Results

Morphological and Molecular Identification of Anopheles species before MALDI-TOF Analysis

All the *Anopheles* specimens sampled were morphologically identified as *An. gambiae* s.l. (n=274) and *An. funestus* gpe (n=21). Only the *gambiae* complex was subjected to molecular characterization using endpoint PCR targeting the IGS region. The primer mixes used generated fragments with theoretical sizes of 221 bp for *An. gambiae* and 333 bp for *An. coluzzii*. *An. gambiae* was the most identified cryptic species, with a proportion of 93% (256/274), compared to *An. coluzzii*, which represented only 1.4% (4/274). The remaining 14 samples failed to amplify. They were retested by endpoint PCR using *An. arabiensis* primers (387 bp), and all were negative. The distribution of *An. gambiae* and *An. coluzzii* is shown in Table 1.

Table 1. Cryptic species of the gambiae complex identified by PCR-IGS

Districts	N analysed	Species identified		Negative
		An gambiae	*An coluzzii*	
Tshangu	95	83	3	9
Funa	60	58	0	2
Mont Amba	79	76	1	2
Lukunga	40	39	0	1
Total (%)	274	256 (93,4%)	4 (1,4%)	14 (5,1%)

Construction of the reference database of protein spectra for An. gambiae s.l. and An. funestus gpe from Kinshasa

The specimens of the two cryptic species of the *gambiae* complex and *An. funestus* gpe (morphologically identified) were subjected to identification by mass spectrometry (MALDI-

TOF). Four spots from each anatomical part of *An. gambiae* s.l. and *An. funestus gpe* were subjected to proteomic analysis by MALDI-TOF. The MALDI Biotyper algorithm (Log Score Value: LSV) allowed the exclusion of several poor-quality spot spectra (LSV < 2) compared to their mean mass spectrum of protein (MSP). 72% of the spot spectra submitted to the *MALDI Biotyper* algorithm had LSV ratios greater than 2. *An. funestus* gpe presented more high-quality spectra (86.1% of LSV > 2) than *An. gambiae* (60.1% of LSV > 2) and *An. coluzzii* (75% of LSV > 2). In general, the proportion of specimens with an LSV > 2 was higher with the heads (405/484) than for the thoraxes (320/484) and legs (324/484). The distributions, by species and anatomical sections, of the different spectra retained (LSV > 2) in the Kinshasa reference database are presented in Table 2.

Table 2. Distribution of Anopheles base spectra retained in the Kinshasa reference database by Anopheles species subjected to MALDI-TOF proteomic analysis

An gambiae (n=96)			
Parties Anatomiques	Spots (n)	Log Score Value	
		LSV < 2	LSV > 2
Heads	384	56	328
Thorax	384	156	228
Legs	384	144	240
(n %) LSV *An.gambiae*	1152	356 (30,9 %)	796 (60,1 %)
An.coluzzii (n= 4)			
Heads	16	4	12
Thorax	16	4	12
Legs	16	4	12
(n %) LSV *An.coluzzii*	48	12 (25,0 %)	36 (75,0 %)
An funestus gpe (n=21)			
Heads	84	19	65
Thorax	84	4	80
Legs	84	12	72
(n%) LSV *An.funestus* gpe	252	35 (13,9 %)	217(86,1%)
Total (n%)	1452 (100,0 %)	403 (27,7 %)	1049 (72,3 %)

Validation of the reference database of protein spectra for different anatomical parts of Anopheles species from Kinshasa

The validation of our reference database was obtained by analysing the spectra of all our samples compared to the reference database obtained using the *MALDI Biotyper* algorithm. The anatomical parts tested for similarity with their counterparts in the constructed spectrum database showed very high recognition scores. The heads recognized heads with a score of 95.43; the thoraxes among themselves, with a score of 90.49; and the legs, with a score of 78.22. The submitted legs and thoraxes poorly recognized the thoraxes or legs in the reference database, with relatively high scores (63.48 and 61.72, respectively) (Table 3).

Table 3. Distribution of identification scores for the different anatomical parts of anopheline species in Kinshasa

Score maximal	Legs identified	Head identified	Thorax identified
Legs identified submitted	**78,22**	44,04	63,48
Head identified submitted	39,7	**95,43**	52,01
Thorax identified submitted	61,72	48,50	**90,49**

Reproducibility of the mass spectra obtained

A strong proximity of the spectra among the species was observed. The spectra of *An. gambiae s.l.* were phylogenetically separated from those of *An. funestus gpe*. Within the *An. gambiae* complex, the two cryptic species, *An. gambiae* and *An. coluzzii*, formed a well-supported monophyletic group but without evidence of a distinction between geographical areas.

Within the *gambiae* complex, the spectral profiles were very similar between the two cryptic species identified. In addition, and regardless of the intensity of spectrum expression, the inter-species protein profiles differed significantly between each anatomical part of the *Anopheles* analysed (Figure 2).

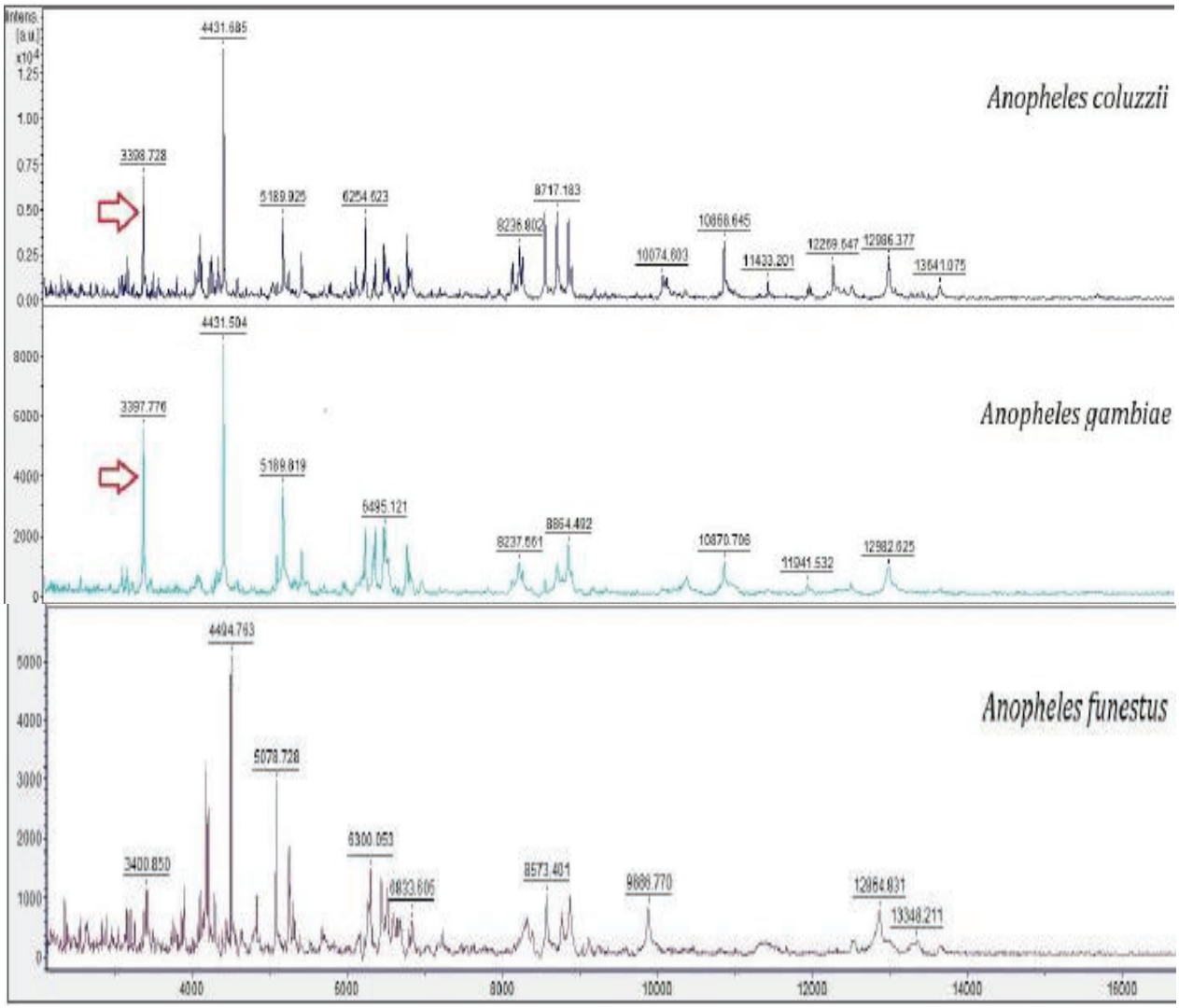

Figure 2. Representative protein profiles of the mass spectra of the different anatomical parts of Anopheles species retained in the Kinshasa reference database (mass/charge ratio: m/z in daltons)

With respect to the various correlation matrices, the levels of reproducibility of the mass spectra are indicated in blue and red, revealing respectively the degree of difference and agreement between the spectra. The small and large yellowish squares represent the correlations *An. funestus gpe x An. funestus gpe* and *An. gambiae* s.l. *x An. gambiae* s.l., respectively. Regardless of the anatomical part and *Anopheles* species considered, the degree of kinship between two identical samples was very strong (diagonal line of the matrix). Significant differences were observed between *An. gambiae* s.l. and *An. funestus* gpe (blue rectangle). The heads showed the best agreement (Figure 3), while the correlations observed among the legs of *An. funestus* gpe and among the thoraxes of *An. gambiae* s.l. showed relatively large differences.

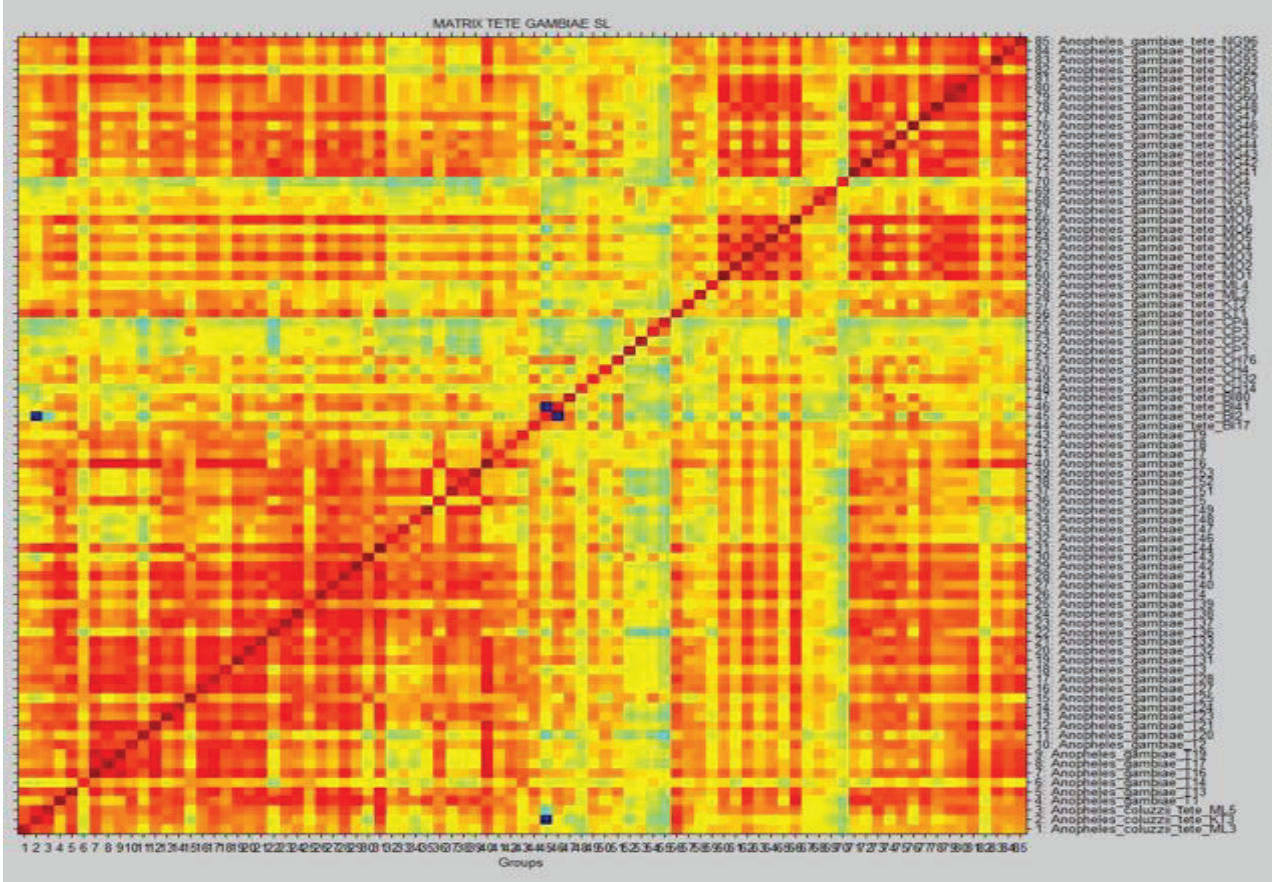

Figure 3: Heat map of the composite correlation index (CCI) of protein profiles from mass spectra of anopheline species circulating in Kinshasa

Identification scores of Anopheles species from Kinshasa

The MSI-2 analysis highlighted the identification scores of *Anopheles* species from Kinshasa and determined the most reproducible anatomical part. After analysing the spectra against the reference database, the recognition scores within the same species were very high. The inter-species recognition score was low between *An. funestus gpe* and the two cryptic species of the *gambiae* complex. High error scores were noted between *An. gambiae* and *An. coluzzii*. No identification errors of anatomical segments of *An. funestus gpe* were observed. The errors were more frequently identified between the legs and thoraxes of the cryptic species of *An. gambiae* s.l. The spectra of the heads of this vector complex showed fewer identification errors.

Discussion

The MALDI-TOF *mass spectrometry is an innovative technique for identifying arthropods.* Its success over the last decade demonstrates that this emerging approach is an important alternative for studying and distinguishing disease vectors circulating in a given geographical area (23). It has many advantages, but the reliability of the results obtained depends on the operating procedure chosen (21-22), the preferred anatomical section of the vector being considered, and the conservation method used (21).

To optimize the use of this technique as an identification tool for the main malaria vectors circulating in Kinshasa, we analysed and evaluated the various parameters to determine the best conservation method to use and the preferred anatomical section to be studied to ensure its success. These data will now serve as reference bases for future *Anopheles* characterization in the region.

To ensure the reliability of the species submitted to *MALDI-TOF*, we performed complementary molecular identifications. Indeed, although time-consuming and very costly in terms of reagents, at present, these

molecular approaches remain the most reliable in terms of identifying *Anopheles* species and subspecies (24). In our study, molecular identification allowed us to characterize the presence of the two cryptic species of the *gambiae* complex (*An. gambiae* and *An. coluzzii*).

The construction of our reference database was carried out using the MS spectra of the two cryptic species of the *gambiae* complex and *An. funestus* gp, morphologically identified, after passing through *MALDI-TOF*. The *An. gambiae s.l.* species analysed from our sample for this study showed the same *MALDI-TOF MS* profiling as those analysed a few months earlier as a trial. It should be noted that the trial samples analysed showed better profiling than those from other African countries maintained in the *PSL Parasitology and Mycology Laboratory* database. As for the *An. funestus* gp spectra, their profilings were similar to those found in the *MALDI-TOF* database of *PSL.*

The *MALDI Biotyper* algorithm excluded 403 of the 1,452 spectra obtained (27.7%) based on their LSV profile < 2. Thus, 72.3% of good quality base spectra (LSV > 2) were retained in our reference database. The general quality of the base spectra is strongly correlated with the approach used for arthropod sampling as well as the methods and durations of conservation before passing through MALDI-TOF *MS* (25-26). Loaiza *et al.* observed that the quality of spectra from *Anopheles* mosquitoes captured in the field was lower than that of colony specimens (25). In the field, mosquitoes can be collected using various capture approaches (*BG Sentinel*, *CDC Light*, human bait captures...). Depending on the deployment time and/or the duration of capture, the samples can be exposed to uncontrolled changes in temperature and relative humidity. As demonstrated by Rakotonirina, the *BG Sentinel* influenced the alteration of the identification score recorded by *MALDI-TOF MS* (26). This alteration was significant after 48 hours of trapping (26).

Comparing the quality of spectra from *Anopheles* mosquitoes collected through various approaches, Nabet noted that field mosquitoes had less clear and homogeneous spectra than laboratory colonies (21). This observation also corroborates that of Nabet, who found that spectra from field-captured specimens showed lower levels of reproducibility than spectra from those reared in the laboratory (21).

Regarding the conservation method, the studys showed that low LSVs were identified in mosquitoes preserved in 70% ethanol, even after short-term conservation. Ethanol promotes the decrease in protein solubility and facilitates a qualitative and quantitative loss of spectral profiles . Rakotonirina in New Caledonia revealed that keeping arthropods at room temperature with silica gel or freezing them prove to be the best methods of mosquito conservation before proteomic analyses (26). The stability of the LSV observed when using silica gel could be linked to its ability to control the relative humidity of samples. However, Diarra in Chad believes that the effectiveness of *MALDI-TOF* in identifying mosquitoes preserved in silica gel is only possible within a two-month conservation period (27).

In our study, we opted for transportation and conservation with silica gel and freezing for more than 6 months. Our percentage of good quality spectra (72.3%) was higher than those obtained by Diarra (61.5%) with *An. gambiae s.l.* preserved in silica gels without freezing (27).

Our conservation method, different from that used by these two authors, may explain the quality of the spectra obtained. The 27% fraction of poor spectra found in our study could be explained by the long freezing period. The storage conditions for transportation to the analysis site (Eppendorf tubes with silica gel kept in cryoboxes, in the airplane hold for an extended period) likely contributed to protein degradation in these samples (28).

Several studies suggest that freezing at -20°C is one of the best conservation methods (21, 28). However, reductions in the best-quality spectra were generally observed after seven months of freezing, with 60% of mosquitoes showing LSVs greater than or equal to the 1.8 threshold (26).

In our study, *An. funestus* gp showed significant proportions of LSV > 2 (86.1%) compared to the members of the *gambiae* complex. The differences in conservation

temperature (-20°C for *An. gambiae s.l.* and -40°C for *An. funestus* gp) are likely the reason for this behavior. Indeed, an additional experiment by Rakotonirina (26). showed that spectra obtained from *Aedes aegypti* stored at -80°C for four years were correctly identified with LSVs greater than 1.8. Storage at temperatures lower than -20°C appears to be the optimal long-term conservation method for use before *MALDI-TOF MS* analysis.

The anatomical sections of *Anopheles* mosquitoes from Kinshasa were individually analysed. Regardless of the species, heads showed more good quality spectra than legs and thoraxes. Our observations align with those of Nabet, who found that interpretable and correct spectra (LSV ≥ 1.7) were more frequent when using the head than the thorax or legs (21). Moreover, the head provided better performance compared to the legs because it did not require the deposition of multiple spots to optimize the log (score) results (21). However, our observations differ from those of Bamou, who observed LSVs higher than the average of 1.8 with thoraxes rather than heads (22). Indeed, several factors related to arthropods (geographical origins, trophic state, or sample homogenization method) can have a moderate or significant impact on the quality of spectra obtained (26,29).

Our sample homogenization method, identical to that of Nabet (21), was not the same as that used by Bamou. In previous studies, the absence of consensus procedures relating to sample preparation contributed to the heterogeneity of results, hampering the comparison and sharing of MS spectra (21,26,29). Visual observation of the MS profiles of the different spectra retained in our study varied depending on the *Anopheles* species and the anatomical sections considered. Regardless of the intensity of spectrum, the profile was identical for the same species and the same anatomical part.

The specificity of visual MS profile spectra is well documented in the literature. Monique Melo Costa noted in her study that MS profiles appear to be specific to both the species and the body part analysed (30). La Roche demonstrated that for the same species, uninfected *Anopheles* mosquitoes showed similar spectral profiles. These profiles differed in infected *Anopheles* mosquitoes (29). Bamou, on the other hand, claimed that among the 720 high-intensity MS spectra tracked, regardless of the mosquito homogenization method, the MSPs were visually reproducible by body part for each species (22). Differences in these MS profiles are also observed in microbiology.

Our results showed that it was nearly impossible to note visual differences in spectra, regardless of the anatomical part, between *An. gambiae* and *An. coluzzii*. This can be explained by the high degree of genetic similarity between these two cryptic species of the *gambiae* complex. Nabet believes that further research is needed to improve the resolution of cryptic species using new bioinformatic data (21).

The ICC matrices of reproducibility of mass protein profiles for three *Anopheles* species from Kinshasa revealed differences between *An. gambiae s.l.* and *An. funestus* gp. Better degrees of kinship were observed between *An. gambiae* and *An. coluzzii*. Regardless of the species, the greatest reproducibility of protein profiles was observed with heads.

The analysis of the MSP dendrograms demonstrated clustering on the same branch for specimens of the same species. The absence of interlacing between species highlights the reproducibility and specificity of protein profiles. The short branch distances confirmed the proximity of their MS spectra. However, within the *An. gambiae* complex, the two cryptic species, *An. gambiae* and *An. coluzzii*, formed a well-supported monophyletic group but without evidence of distinction between geographical areas.

Several studies have demonstrated the species-specificity of dendrograms as well as high ICC values for MS spectra of the same species (22,31). Bamou even asserts that despite the homogenization method, all samples of the same mosquito species were grouped on the same branch, reflecting the reproducibility of spectra with MALDI-TOF *MS* (22).

The validation of our *Anopheles* protein profile spectra database from Kinshasa involved comparing the spectra of interest with those retained in the reference database. For LSV > 2, recognized as the interpretable identification

result threshold in our study, homologous anatomical parts within a given species complex showed very high recognition scores. Heads recognized heads with a score of 95.43; thoraxes among themselves, a score of 90.49, and legs, a score of 78.22.

Poor recognition of anatomical parts with high scores was noted when crossing legs with thoraxes (63.48 and 61.72 vice versa). Poor recognition of heads with other anatomical sections was relatively low. The overall sensitivity of similarity between *Anopheles* mosquitoes from Kinshasa, identified by molecular or morphological methods compared to MALDI-TOF spectra retained in the reference database, varied depending on the species. Very high scores were noted within the same species (95.43, 90.49, and 70.43 for *An. gambiae*, *An. funestus* gp, and *An. coluzzii* respectively). The crossing of *An. funestus* and the two cryptic species of the *gambiae* complex showed very low recognition scores. Moreover, identification error scores between *An. gambiae* and *An. coluzzii* can be explained by their genetic closeness. The thoraxes and legs were more implicated than the heads in the occurrence of these errors.

Identification scores remain variable depending on the arthropods, the geographical origins of the species to be analysed, the LSV limits retained, the quality of treatments, and the anatomical sections of the specimens. Monique Melo Costa, working on *Culex*, obtained relevant identification rates (LSV > 1.8) of around 95% for each of the species analysed (29). Using *An. gambiae* from Mali, Nabet found correct identification of 61.54% for heads compared to only 21.15% for thoraxes and 30.77% for legs. With *An. gambiae* from Guinea, the results were 67.50%, 35% and 27.5% respectively (21). Chavy's observations with *Phlebotomus* demonstrate the influence of LSV on the agreement between identification results by MALDI-TOF *MS* and molecular biology. A threshold below 1.7 would have decreased specificity and increased the risk of producing an incorrect identification result (31).

Our study has shown that MALDI-TOF *MS* seems to be an effective tool for identifying *Anopheles* mosquitoes stored cold with silica gel. Although the number of samples used in our study was not very large, the results obtained, namely the high rate of good quality spectra with a high LSV (compared with other studies), the visual reproducibility of the spectra by species, the incongruity between two different species, and the interesting identification scores in relation to the anatomical section used, are robust and therefore pave the way for future studies with a larger number of samples to confirm these preliminary results.

Apart from cryptic species, there is theoretically no limit to the identification capacity of the MALDI-TOF *MS* technique as long as the database contains enough adequate reference spectra. Compared to PCR, a hundred spots from our MALDI-TOF *MS* analyses were evaluated in a few hours, whilst the molecular method required several rounds of treatment and preparation, ranging from time-consuming DNA extraction to agarose gel migration.

The initial cost of a MALDI-TOF system is high, including the instrument and analysis software. However, the cost per sample is very low once the equipment has been purchased, making it an economically viable solution for processing large sample volumes (21). In contrast, molecular approaches require a lower initial investment. However, the cost per sample is high (5). This cost includes reagents, specific primers and DNA extraction kits, which can make this approach more expensive in the long term for mass identification.

Conclusion

The present study suggests that MALDI-TOF MS is an effective method for the rapid and accurate identification of *Anopheles* species in Kinshasa. The technique shows good reproducibility of protein spectra, particularly with mosquito heads. Validation of the mass spectra database for different malaria species in Kinshasa shows excellent reliability for specimen identification, with high scores for heads and a good overall recognition rate. Compared with molecular techniques used for the identification of *Anopheles*, MALDI-TOF MS offers several advantages, including a reduction in analysis time, which can facilitate the examination of a large number of samples.

This database will serve as a reference for future entomological studies in the region and

could be used to strengthen malaria vector surveillance programmes.

Conflict of interest

No competing interests have been declared by the authors.

Contribution of the authors

Josue Zanga and Roger Wumba were responsible for the design and implementation of the study. Josue Zanga, Osee Mansiangi, Victoire Nsabatien, Maxwel Bamba, Bienvenue Lutumbu, Vanessa Mvudi and Freddy Arnold Kabasele were in charge of data collection. JZ, Fiacre Agossa, Emery Metelo and Nono Mvuama performed the statistical analysis and prepared the manuscript for publication. All authors were involved in drafting the manuscript. Funding for the study was provided entirely by the Laboratoire de Bioécologie et de Lutte Antivectorielle of the Ecole de Santé Publique in Kinshasa.

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Cite this article as : Zanga J, Nsabatien V, Mvudi V, Kabasele FA, Agossa F, Mvuma N, *et al.* Contribution of MALDI-TOF to the identification of Anopheles gambiae complexes in Kinshasa,

Democratic Republic of Congo. *Ann Afr Med* 2025; **19** (1): e6592-e6606. https://dx.doi.org/10.4314/aamed.v19i1.2

Digital media overuse may be associated with poor cognition among school-age children in Kinshasa, Democratic Republic of Congo

Usage excessif d'écrans numériques et faible cognition chez les enfants en âge de scolarité à Kinshasa, République Démocratique du Congo

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