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Identification of mixed and successive blood meals of mosquitoes using MALDI-TOF MS protein profiling

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Research Article

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Abstract

Background. The accurate and rapid identification of mosquito blood meals is critical to study the interactions between vectors and vertebrate hosts and, subsequently, to develop vector control strategies. Recently, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) profiling has been shown to be a reliable and effective tool for identifying single blood meals from mosquitoes.

Methods. In this study, we developed MALDI-TOF MS profiling protocols to identify *Anopheles gambiae* Giles, *Anopheles coluzzii* and *Aedes albopictus* mosquitoes' mixed blood meals and the last of successive blood meals. The mosquitoes were either successively artificially fed with distinct host bloods or engorged with mixed bloods from distinct vertebrate hosts, such as humans, sheep and dogs.

Results. Blind test analyses revealed a correct identification of mixed blood meals from mosquitoes using MALDI-TOF MS profiling. The 353 MS spectra from mixed blood meals were identified using log score values >1.8. All MS spectra ($n = 244$) obtained from mosquitoes' successive blood meals were reproducible and specific to the last blood meal, suggesting that the previous blood meals do not have an impact on the identification of the last one.

Conclusion. MALDI-TOF MS profiling approach appears to be an effective and robust technique to identify the last and mixed blood meals during medical entomological surveys.

Introduction

Mosquitoes are the major arthropod vectors of human infectious diseases across the world (Becker *et al.*, 2010). *Aedes* mosquitoes are able to transmit arboviral diseases including Yellow Fever, Dengue, Chikungunya and Zika viruses (Gardner and Ryman, 2010; Vasilakis *et al.*, 2011; Caglioti *et al.*, 2013; Gould *et al.*, 2017). As for *Anopheles* mosquitoes, they include the main vectors of malaria (Carnevale *et al.*, 2009). Malaria is the primary cause of morbidity and mortality in Africa, mainly amongst the children under the age of 5 and pregnant women (WHO, 2016). There is still no effective vaccine for several mosquito-borne diseases; entomological surveys and vector control measures are hence essential to combat them (Deilgat *et al.*, 2014).

Mosquito vectors may feed on multiple hosts, including humans but also various domesticated or wild animals. This may result in successive or interrupted blood meals. A better understanding of the trophic preferences of mosquito vectors is an important factor to evaluate the risk of transmission of zoonotic diseases (Githeko *et al.*, 1994; Shililu *et al.*, 1998; Coulibaly *et al.*, 2016; Ndenga *et al.*, 2016). Studies on the interaction between *Anopheles* malaria vectors and their trophic preferences are critical to assess the risk of human exposure to *Plasmodium* parasites (Fyodorova *et al.*, 2006). In Senegal, when the blood meals of 1886 freshly fed *Anopheles gambiae* Giles were studied, it was shown that most blood meals had been taken on a single host (40.1% on humans and 37.1% on animals). Mixed blood meals were mostly human–animal mix than non-human strictly (Ngom *et al.*, 2013). In Kenya, mixed blood meals were shown to be predominant in culicine mosquitoes such as *Culex quinquefasciatus*. Up to 94% of the mosquitoes, according to the species, had fed on different hosts, including humans and Rift Valley Fever animal hosts (Muturi *et al.*, 2008). Other reports highlighted that *Anopheles* mosquitoes fed both on humans and their domestic animals such as cattle and goats (Muriu *et al.*, 2008). The identification of vertebrate hosts in mixed blood meals of mosquitoes may also be critical to adapt control strategies. For example, in 2016, an unusually high frequency of animal and mixed human–animal blood meals in the major malaria vector *An. gambiae s.s.* was revealed in the western Kenya Highlands. The authors suggested that this was linked with the vectors' response to increased bed net coverage in the

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area and the close location of livestock as people at night, opening the door for livestock-targeted interventions for malaria control (Ndenga *et al.*, 2016).

Several methods are used to determine the origin of mosquito blood meals, including precipitin tests, counter-current immunoelectrophoresis and enzyme-linked immunosorbent assays (ELISA) (Fyodorova *et al.*, 2006; Thomas *et al.*, 2017). Serological tests are affordable and widely used but limited by the availability of antibodies for uncommon species (Beier *et al.*, 1988) and samples that remain non-reactive (Mucci *et al.*, 2015), for example.

Molecular approaches based on host DNA amplification can be highly specific (Prior and Torr, 2002; Kent and Norris, 2005; Kent, 2009; Munnoz *et al.*, 2011; Logue *et al.*, 2016; Ndenga *et al.*, 2016), although they are curbed by their running costs, turnaround time and the comprehensiveness of online sequence databases such as NCBI GenBank (Oshaghi *et al.*, 2006; Egizi *et al.*, 2013).

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a protein profiling-based technique that uses a laser energy absorbing matrix to generate a protein spectrum from an organism of interest (Seng *et al.*, 2010). It has revolutionized clinical microbiology by making it possible to rapidly identify bacteria at a low cost (Seng *et al.*, 2009, 2013; Schubert and Kostrzewa, 2017). In recent years, the effectiveness of MALDI-TOF MS for identifying arthropods (Tandina *et al.*, 2016, 2018a; Yssouf *et al.*, 2016; Diarra *et al.*, 2017; Laroche *et al.*, 2017*b*; Halada *et al.*, 2018; Mewara *et al.*, 2018) and mosquito blood meals identification (Niare *et al.* 2016, 2017*b*; Tandina *et al.*, 2018*a*,*b*) has been reported. Indeed, in preliminary studies, MALDI-TOF MS was able to identify the host blood source from *An. gambiae* Giles and *Aedes albopictus* mosquitoes artificially fed on human, horse, sheep, rabbit, mouse, rat and dog blood up to 24 h post-feeding (Niare *et al.*, 2016, 2017b; Tandina *et al.*, 2018*b*).

The goal of the present study was to determine whether MALDI-TOF MS could be extended to the identification of mixed and successive blood meals.

Anopheles gambiae Giles, *Anopheles coluzzii* and *Ae. albopictus* mosquitoes were artificially engorged on human blood and subsequently fed on the blood of other vertebrates after complete digestion of the first meal. For the identification of mixed blood meals by MALDI-TOF MS, the mosquitoes were engorged with blood mixed at different concentrations from different vertebrate hosts to mimic interrupted blood meals.

Materials and methods

Ethical statement

Animal studies were conducted in line with Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010, and in compliance with French Government Decree No. 2013-118 of 1 February 2013. The study was approved by the local Animal Ethics Committee (Comité d'Ethique en Expérimentation Animale, Marseille) and the Institutional Animal Care Committee in Marseille, France. Human blood was obtained from the national French blood bank, the 'Etablissement Français du Sang' (EFS) accredited by the Institutional Animal Care Committee of IHU Méditerranée Infection.

Mosquito rearing and maintenance

Anopheles gambiae Giles, *An. coluzzii* and *Ae. albopictus* mosquitoes were reared in our laboratory (Marseille, France) using standard methods at a temperature of 26 ± 1°C, a relative humidity of 80 ± 10% and a 12 h photoperiod in incubators (Panasonic cooled incubator) (Awono-Ambene *et al.*, 2001). *Anopheles gambiae* Giles and *An. coluzzii* colonies were established from laboratory colonies from the 'Institut de Recherche pour le Développement' (IRD, Montpellier, France) and IRD Dakar, Senegal, respectively. *Aedes albopictus* mosquitoes were originally sampled at the larval stage in a pond in Marseille, France in 2011 and reared in our laboratory.

Larvae were reared until the pupal stage in trays containing 1 L of distilled water supplemented with fish food (TetraMinBaby, Tetra Gmbh, Herrenteich, Germany). Pupae were collected daily and transferred into a mosquito cage (Bug Dorm 1 insect rearing cage, BioQuip products, Taiwan). Adults were fed with a 10% glucose solution until the day of the experiment. Three days after emergence, female adult mosquitoes were artificially fed through a Parafilm-membrane (Hemotek membrane feeding systems, Discovery Workshops, UK) using fresh heparinized human blood for 2 h. Engorged female mosquitoes were transferred to another cage and were fed with a 10% glucose solution on cotton.

Successive blood meals

To assess the ability of MALDI-TOF MS to identify the last blood meal taken, 60 *An. gambiae* Giles, 60 *An. coluzzii* and 124 *Ae. albopictus* were firstly engorged on human blood. Only one host donor was used per species to limit intraspecific variability. Twelve hours after feeding, 10 *Anopheles* and 20 *Aedes* abdomens were submitted to MALDI-TOF MS analysis, while, for each mosquito species, the remaining specimens were dispatched equally in six cages. Three days after the first blood meal (after complete digestion), the mosquitoes from each cage were engorged for a second time on six distinct types of vertebrate blood (goat, cow, chicken, dog, sheep and rabbit). The abdomens of all fully engorged mosquitoes were submitted to MALDI-TOF MS analysis 12 h after feeding.

Mixed blood meals

To determine the ability of MALDI-TOF MS to identify mixed blood meals from mosquito abdomens, the mosquitoes were fed on human blood mixed with dog blood or sheep blood at ratios of 75/25%, 50/50% and 25/75% (Table 1). To prevent the coagulation of blood mixtures, the various mixtures of blood were previously heated at 56°C in a dry bath for 30 min, as previously described (Nossel and Niemetz, 1965). Only one host donor was used per species to limit intraspecific variability.

The mosquitoes' abdomens were collected 12 h after feeding and then submitted to MALDI-TOF-MS analysis. Human, sheep and dog blood was mixed at equal concentrations and given to 25 *An. gambiae* Giles, 10 *An. coluzzii* and 23 *Ae. albopictus* (Table 1).

A flowchart illustrating the main steps in the experimental workflow for the arthropod spectra database creation and blood meal identification by MALDI-TOF MS is presented in Fig. 1.

Sample preparation for MALDI-TOF MS analysis

Only fully engorged mosquitoes were included in the MS analysis. Engorged females were killed at −20°C prior to dissection. Each mosquito abdomen was separated from the thorax using a sterile scalpel for each sample. The dissected abdomens were manually crushed using sterile pestles (Fischer Scientific, Strasbourg, France) in a 1.5 mL tube containing 50 μL of HPLC-grade water. Ten microlitres of the supernatant of the crushed abdomens were homogenized in 20 μL of 70% formic acid and 20 μL

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Table 1. Number of mosquito samples submitted to MS analysis

	Concentration	Samples	*Anopheles gambiae*	*Anopheles coluzzii*	*Aedes albopictus*	Total
Mixed blood meals Human–Dog	75%Human/25% Dog	Number of samples	14	10	23	47
		Number of specimens for DB upgrading	/	1	2	3
	50%Human/50% Dog	Number of samples	14	10	23	47
		Number of specimens for DB upgrading	4	1	3	8
	25%Human/75% Dog	Number of samples	14	10	23	47
		Number of specimens for DB upgrading	/	1	2	3
Mixed blood meals Human–Sheep	75%Human/25% Sheep	Number of samples	4	10	22	36
		Number of specimens for DB upgrading	/	2	2	4
	50%Human/50% Sheep	Number of samples	5	14	23	42
		Number of specimens for DB upgrading	5	2	3	10
	25%Human/75% Sheep	Number of samples	4	13	23	40
		Number of specimens for DB upgrading	/	2	2	4
Mixed blood meals Human–Dog–sheep	Equal concentration	Number of samples	20	10	23	53
		Number of specimens for DB upgrading	5	1	3	9
Total	/	/	89	87	177	353

of 50% acetonitrile (Fluka, Buchs, Switzerland) and centrifuged at 10 000 rpm for 20 s. One microlitre of supernatant of each sample was deposited on the MALDI-TOF target plate in quadruplicate (Bruker Daltonics, Wissembourg, France) and covered with 1 μL of CHCA matrix solution composed of saturated α-cyano-4-hydroxycynnamic acid (Sigma, Lyon, France), 50% acetonitrile (v/v), 2.5% trifluoroacetic acid (v/v) (Sigma) and HPLC-grade water. After drying for several minutes at room temperature, the target was introduced into the Microflex LT MALDI-TOF Mass Spectrometer (Bruker Daltonics, Germany) for analysis. To control loading on mass spectra steel, matrix quality and MALDI-TOF MS apparatus performance, the matrix solution was loaded in duplicate onto each MALDI-TOF MS plate with or without a bacterial test standard (Bruker protein Calibration Standard I), as previously described (Niare *et al.*, 2016).

MALDI-TOF MS parameters

Protein mass profiles were acquired using a Microflex LT MALDI-TOF Mass Spectrometer, with detection in the linear positive-ion mode at a laser frequency of 50 Hz within a mass range of 2–20 kDa. The acceleration voltage was 20 kV, and the extraction delay time was 200 ns. Each spectrum corresponded to ions obtained from 240 laser shots performed in six regions of the same spot and automatically acquired using the AutoXecute function of the Flex Control v.2.4 software (Bruker Daltonics, Germany). The spectrum profiles obtained were visualized using Flex analysis v.3.3 software and exported to ClinProTools version v.2.2 (Bruker Daltonics, Germany) and MALDI-Biotyper v.3.0. (Bruker Daltonics, Germany) for data processing (smoothing, baseline subtraction and peak picking) and evaluation with cluster analysis.

MALDI-TOF MS spectra analyses

The reproducibility and specificity of MALDI-TOF MS spectra from the abdomens of mosquitoes which had taken successive or mixed blood meals were evaluated by comparing the four spectra of each sample tested using the Flex Analysis and ClinProTools 2.2 software. The gel view tool of ClinProTools 2.2 software was used to control intraspecific reproducibility. As a control, 1 μL of host blood was mixed with 20 μL of 70% formic acid and 20 μL of 50% acetonitrile and deposited on the MALDI-TOF target plate in quadruplicate and covered with 1 μL of CHCA matrix solution.

The spectra from *An. gambiae* Giles mixed blood meals of human/dog blood ($n = 4$), human/sheep blood ($n = 5$) and the triple mixture of human/dog/sheep blood ($n = 5$) were selected to upgrade our arthropod spectra database (Table 1). As for *Ae. albopictus*, the reference spectra of human/dog blood ($n = 7$), human/sheep blood ($n = 7$) and the triple mixture of human/dog/sheep blood ($n = 3$), and *An. coluzzii* the reference spectra of human/dog blood ($n = 3$), human/sheep blood ($n = 6$) and the triple mixture of human/dog/sheep blood ($n = 1$) were selected to upgrade the database (Table 1). All these 41 spectra were selected for the database on the basis of their high intensity, reproducibility and global spectrum quality. Details of all spectra selected to upgrade the database are presented in Table 1. This database includes the spectra obtained from various arthropod species and mosquitoes' abdomens fed on 25 distinct host bloods (*Homo sapiens*, *Equus caballus*, *Ovis aries*, *rabbit*, Balb/C mouse, *Rattus norvegicus*, *Canis familiaris*, *Bos taurus*, *Capra hircus*, *Gallus gallus*, *Equus asinus*, *Tapirus indicus*, *Tapirus terrestris*, *Carollia perspicillata*, *Thraupis episcopus*, *Erythrocebus patas*, *Callithrix pygmaea*, *Leopardus pardalis*, *Arctictis binturong*, *Antidorcas marsupialus*, *Panthera onca*, *Papio hamadryas*, *Camelus dromedarius*, *Ammotragus lervia* and *Sus scrofa)* (Additional file: Table S1).

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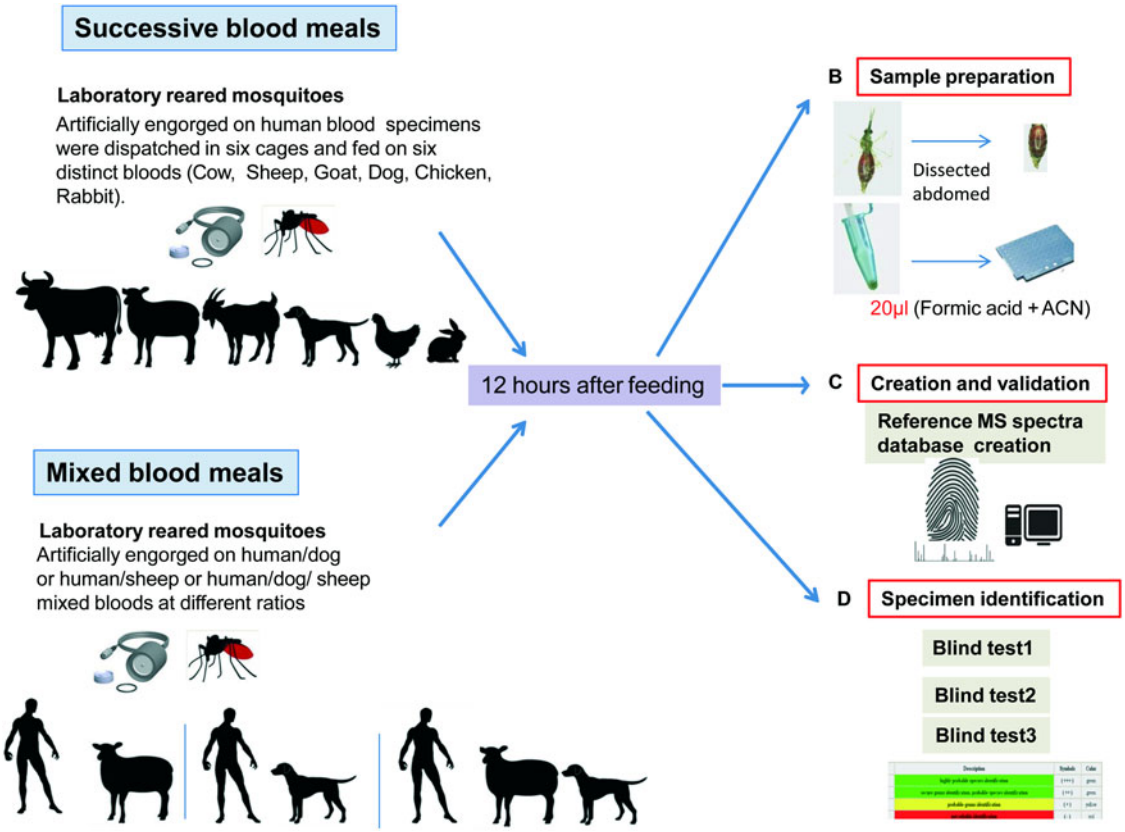

Fig. 1. Experimental workflow for mixed and successive blood meal identification using MALDI-TOF MS.

Main Spectrum Profiles (MSPs) were automatically created from the four spectra per sample based on an algorithm using the information on peak position, intensity and frequency from the BioTyper MSP Creation Standard Method. The maximum mass error of each single spectrum was 2000 Da, and the desired mass error for the MSP was 200 Da.

Blind tests

Blind test 1

The abdomen-derived spectra from *An. gambiae* Giles which had taken successive blood meals were submitted for a blind test analysis. Similarly, the spectra of the abdomens of the mosquitoes fed with mixed blood meals (without those selected to be entered in the MS database) were subjected to a blind test analysis against the database (DB1) (Table 1). The results are presented in the MALDI-Biotyper software v.3.3. as log score values (LSVs) that consists of the degree of signal intensity matching between the queried mass spectra and the reference spectra. LSVs range from 0 to 3. LSVs allow a good assessment of the reproducibility between a queried spectrum and a reference spectrum, as they are the result of the thorough comparison of peak positions and intensity between those two spectra (MALDI BioTyper Help, Bruker). An LSV was obtained for each spectrum of the blind tested samples. The samples were considered to be correctly and significantly identified when the spectrum queried had an LSV ≥1.8 (Laroche *et al.*, 2017*a*; Niare *et al.*, 2016).

Blind test 2

MS spectra obtained from engorged abdomens of *Ae. albopictus* and *An. coluzzii* after successive and mixed blood meals were queried against the arthropod spectra database upgraded with reference spectra of *An. gambiae* Giles (DB1). *Aedes albopictus* and *An. coluzzii* reference spectra (Table 1) for successive and mixed blood meals were then added to DB1 to create DB2 (Additional file: Table S2).

Blind test 3

The spectra of the abdomens of the *Ae. albopictus* and *An. coluzzii* fed with mixed blood meals (with the exception of those selected to be entered in the database) were tested against DB2. An LSV was obtained for each of the four spots of each specimen tested. A flowchart of blind test analyses is available as supplementary data (Additional file: S3)

Results

Successive blood meals

Comparison of the MS spectra from *An. gambiae* Giles ($n = 60$), *Ae. albopictus* ($n = 124$) and *An. coluzzii* ($n = 60$) abdomens after successive blood meals using the Flex Analysis software indicated a high quality (Fig. 2) and reproducibility of the spectra, allowing the inclusion of all spectra in the study. Querying *An. gambiae* Giles against DB1 (Additional file: Table S1) after successive blood meals, showed reliable and 100% accurate identification of the last blood meal ($n = 60$) (Table 2). The LSVs for the specimens ranged between 1.826 and 2.764. According to preliminary studies, the samples are considered to be correctly and significantly identified when the spectrum queried had an LSV ≥1.8 (Niare *et al.*, 2016; Laroche *et al.*, 2017*a*). The spectra from the engorged abdomens of *Ae. albopictus* and *An. coluzzii* were then queried against DB1. We observed 80% correct identification with a mean score of 2.242. When tested against DB2, 100% of the blood meals were correctly identified, with a mean score of 2.593 (Table 3). The LSVs for the specimens ranged between 1.855 and 2.873.

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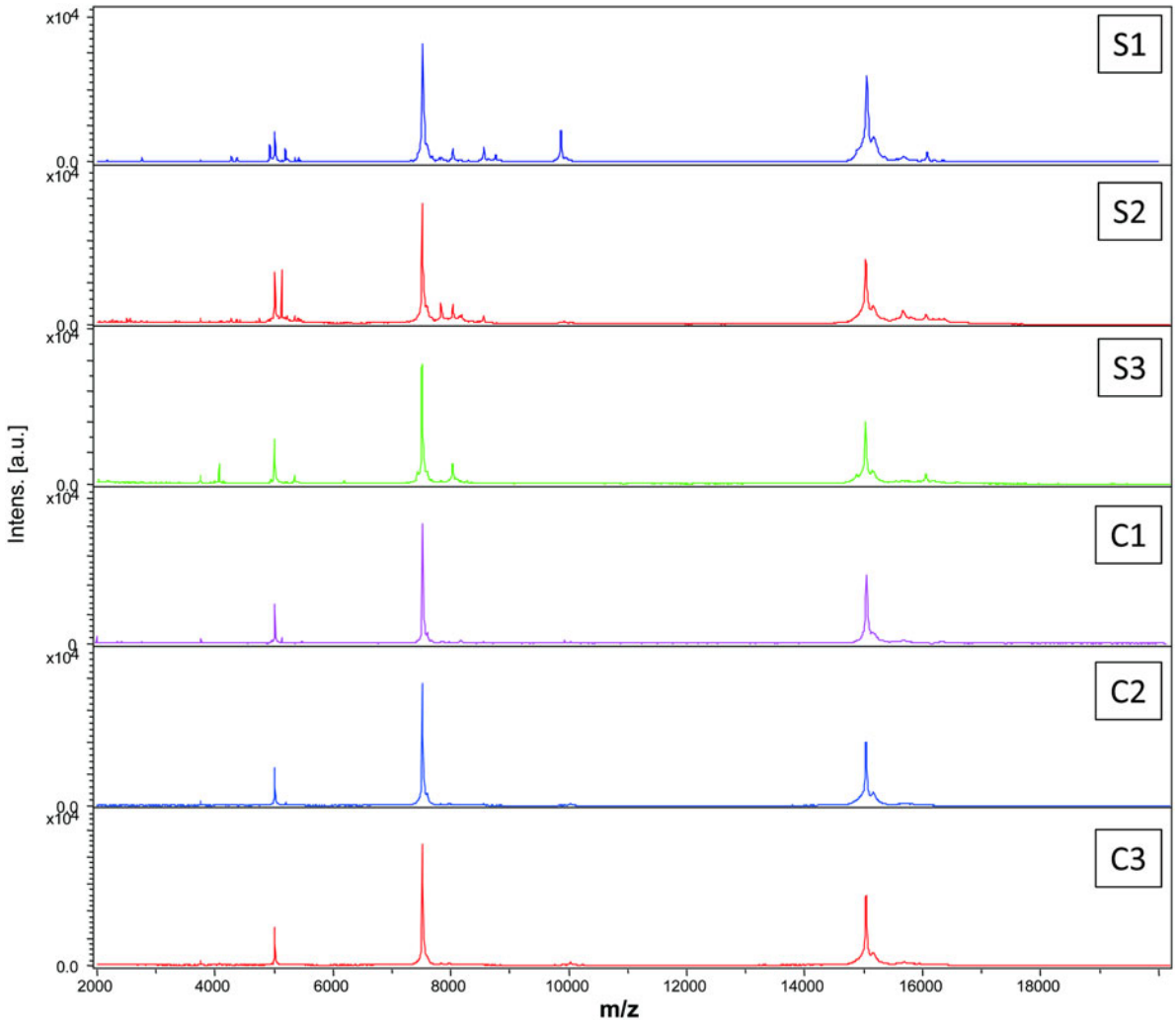

Fig. 2. Representative abdomen-derived MALDI-TOF MS spectra from *Anopheles gambiae* Giles, *Anopheles coluzzii* and *Aedes albopictus* fed on sheep (S1,S2,S3) and cow (C1,C2,C3). All mosquitoes were collected 12 h post-feeding. Au = arbitrary units; m/z = mass-to-charge ratio.

Mixed blood meal identification by MALDI-TOF MS

Regarding the human/dog and human/sheep blood mixtures, a correct identification was observed for all 25 *An. gambiae* Giles mosquitoes that fed on mixed blood meals, with LSVs ranging from 2.055 to 2.771 (Table 4).

The query of spectra from engorged abdomens of *Ae. albopictus* and *An. coluzzii* against DB1 containing reference spectra of engorged *An. gambiae* Giles only revealed 51.51% and 51.42% correct identification of blood mixtures from human/dog and human/sheep blood, respectively. After upgrading the database to DB2, we obtained accurate identification for 100% of the blood meals, with LSVs ranging from 1.762 to 3 (Table 5 for human/dog mixed blood meals, Table 6 for human/sheep mixed blood meals).

Finally, the 20 *An. gambiae* which had been fed with a triple mixture of blood (human/dog/sheep) were subjected to MS analyses. Their abdomens provided good quality spectra and the following blind test led to unambiguous, accurate identification with LSVs ranging from 2.258 to 2.846 (Table 4). *Aedes albopictus* and *An. coluzzii* mosquitoes fed with the same triple mixture revealed 36.36% correct identification when blind tested against DB1. After upgrading the database, we observed 100% correct identification of the blood meals with LSVs ranging from 2.070 to 2.725 in the blind test against DB2 (Table 7).

Discussion

MALDI-TOF MS consists of three major steps. Firstly, the organism of interest is mixed with a suitable matrix material and applied to a metal plate. Secondly, the co-crystallized sample is irradiated with laser pulses, effecting desorption and 'soft' ionization of the organism. Thirdly, molecules are accelerated in an electric field and separated through a flight tube in linear or reflectron mode, according to their mass-to-charge ratio. The MS protein profiles obtained are used to discriminate between each specimen tested (Yssouf *et al.*, 2016).

MALDI-TOF MS has been shown to be reliable for the identification of single blood meals both in laboratory and field conditions (Tandina *et al.*, 2018*a*; Tandina *et al.*, 2018*b*). The purpose of the present study was to artificially mimic what happens in the field when a blood meal is completed on another animal host before (mixed blood meal design) or after (successive blood meals design) digestion of the first meal. Livestock and domestic animals were included in the study as they can be the source of several vector-borne pathogens such as Rift Valley Fever (RVF) (Linthicum *et al.*, 2016), Crimean-Congo haemorrhagic virus (CCHV) (Bente *et al.*, 2013), ehrlichiosis (Unver *et al.*, 2001) and tularaemia for rabbits (Petersen *et al.*, 2009). In this proof-of-concept, only mosquitoes were included but further study is conceivable to assess the ability of MALDI-TOF MS to successfully identify the blood meals of other arthropods of medical and veterinary interest.

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Table 2. MALDI-TOF MS identification of *An. gambiae* successive blood meals

Mosquito species	First blood meal	Second blood meal	Number of specimens used for blind test	Results of MS identification [range LSVs]
An. gambiae	Human	/	10	Human [1.834–2.320]
An. gambiae	Human	Goat	10	Goat [2.173–2.670]
An. gambiae	Human	Dog	10	Dog [2.139–2.764]
An. gambiae	Human	Cow	10	Cow [1.810–2.396]
An. gambiae	Human	Sheep	10	Sheep [1.802–2.296]
An. gambiae	Human	Rabbit	10	Rabbit [1.826–2.101]
Total	/	/	60	

All mosquitos were firstly engorged on human blood and then fed on goat, dog, cow, sheep and rabbit blood.

Table 3. MALDI-TOF MS identification of successive blood meals of *Aedes albopictus* and *Anopheles coluzzii*

Mosquito species	First blood meal	Second blood meal	Identification of blood origin before upgrade (DB 1)	LSVs obtained before upgrade	Number of specimens used for DB upgrade	Identification of blood origin after upgrade (DB2)	LSVs obtained after upgrade
Ae. albopictus	Human	/	*Ae. albopictus* Human (22)	[2.148–2.602]	/	*Ae. albopictus*-Human(22)	[2.148–2.602]
Ae. albopictus	Human	Cow	*An. gambiae*-Cow (12)	[1.889–2.387]	3	*Ae. albopictus*-Cow(17)	[1.941–2.873]
			An. coluzzii-Mouflon (8)	[2.225–2.645]			
Ae. albopictus	Human	Sheep	*An. gambiae*-Sheep(2)	[1.714–1.745]	3	*Ae. albopictus*-Sheep(17)	[1.972–2.754]
			An. coluzzii-Mouflon(18)	[1.749–2.719]			
Ae. albopictus	Human	Goat	*An. gambiae*-Goat(13)	[1.762–1.961]	3	*Ae. albopictus*-Goat(17)	[2.206–2.752]
			An. coluzzii-Mouflon(5)	[2.085–2.492]			
			Insufficient(2)	[1.549–1.696]			
Ae. albopictus	Human	Chicken	*An. gambiae*-Chicken (20)	[1.894–2.294]	2	*Ae. albopictus*-Chicken(18)	[2.117–2.636]
Ae. albopictus	Human	Rabbit	*An. gambiae*-Rabbit(21)	[1.429–1.843]	3	*Ae. albopictus*-Rabbit(19)	[2.196–2.535]
			An. gambiae-Ocelot(1)	[1.767]			
An.cColuzzii	Human	/	*An. gambiae*-Human(10)	[1.964–2.421]	1	*An. coluzzii*-human(9)	[2.217–2.421]
An. coluzzii	Human	Cow	*An. gambiae*-Cow (10)	[1.648–2.543]	1	*An. coluzzii*-Cow(9)	[2.125–2.543]
An. coluzzii	Human	Sheep	*An. gambiae*-Sheep(10)	[1.836–2.584]	1	*An. coluzzii*-Sheep(9)	[1.855–2.584]
An. coluzzii	Human	Goat	*An. gambiae*-Goat (10)	[1.637–1.943]	1	*An. coluzzii*-Goat(9)	[2.014–2.239]
An. coluzzii	Human	Chicken	*An. gambiae*-Chicken(4)	[1.236–1.38]	1	*An. coluzzii*-Chicken(9)	[1.948–2.819]
			An. gambiae-Ocelot(4)	[1.286–1.373]			
			An. gambiae-Cow(1)	[1.467]			
			An. gambiae-Goat(1)	[1.341]			
An. coluzzii	Human	Rabbit	*An. gambiae*-Rabbit(10)	[1.581–1.851]	1	*An. coluzzii*-Rabbit(9)	[2.120–2.643]
Total			184	80%	20	164	100%

[(x)]Number of specimens included in each analysis.
DB, database

MALDI-TOF MS was also able to successfully identify mosquito blood meals up to 24 h post feeding using engorged abdomens (Niare *et al.*, 2016; Tandina *et al.*, 2018*b*). This time point may limit MS identification but Sella's index allows selection of fully engorged mosquitoes, which can be numerous when caught in houses in the morning or in early-collected traps (Tandina *et al.*, 2018*a*). The quality of the spectra is stable until blood digestion begins. Spectra from freshly but partially engorged mosquitoes should be identifiable too using MALDI-TOF MS but may not be stable up to 24 h.

Artificial feeding laboratory conditions will probably never mimic the real conditions of mosquitoes feeding in the field or the reality of variable stages of blood digestion at the time of mosquito sampling. However, the MALDI-TOF MS approach was previously validated on field samples collected in Comoros and Mali (Niare *et al.*, 2017*a*; Tandina *et al.*, 2018*a*).

The 24 h limit for relevant identification can be circumvented by picking up the mosquito traps early. The blood meals were correctly identified from mosquitoes' abdomens crushed on Whatman filter papers (WFP) by MALDI-TOF MS (Niare *et al.*, 2017*a*; Tandina *et al.*, 2018*a*).

However, some studies have shown that mixed or multiple blood meals can amount to up to 10% of the mosquitoes in the field (Logue *et al.*, 2016; Moreno *et al.*, 2017).

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Table 4. MALDI-TOF MS identification of *Anopheles gambiae* Giles mixed blood meals

Mosquito species	Concentration of blood meal	Number of Specimens for 1st BT	Results of MS identification before DB upgrade [scores]	Number of specimens used for DB upgrade	Results of MS identification after upgrade (DB 1) [scores]
Anopheles gambiae	75%Human/25%Dog	14	14/14 Human [1.819–2.23]	/	14/14 Human/Dog [2.055–2.529]
	50%Human/50%Dog	14	9 Human + 5 Dog [1.828–2.004]	4	10/10 Human/Dog [2.115–2.477]
	25%Human/75%Dog	14	10/10 Dog [1.615–2.017]	/	14/14 Human/Dog [2.161–2.614]
	75%Human/25%Sheep	4	3 Human + 1 Sheep [1.897–2.047]	/	4/4 Human/Sheep [2.399–2.493]
	50%Human/50%Sheep	5	5/5 Sheep [1.146–2.216]	5	N/A◆
	25%Human/75%Sheep	4	4/4 Sheep [2.09–2.372]	/	4/4 Human/Sheep [2.405–2.771]
	Human/Dog/Sheep Equal concentration	20	6 Human + 4 Human/Dog + 10 Human Sheep [1.791–2.38]	5	15/15 Human/Dog/ Sheep [2.258–2.846]
Total		75		14	61/61 correct identifications

DB, database; BT, blind test.
◆No spectra blind tested because all were used as reference.

Table 5. Influence of mosquito species on MALDI-TOF MS identification of *Aedes albopictus* and *Anopheles coluzzii* fed on human/dog mixed blood meals human/dog mixed blood meals

Mosquito species	Concentration of blood meal	Results of MS identification before upgrade (DB 1)	LSVs obtained before upgrade	Number of specimens used for DB upgrade	Results of MS identification after upgrade (DB 2)	LSVs obtained after upgrade
Ae. albopictus	75%Human/25% Dog	*An. gambiae*-Human–Dog (2)	[1.739–1.763]	2	*Ae. albopictus*-Human–Dog (21)	[1.762–2.665]
		Ae. albopictus-Human(20)	[1.700–2.140]			
		Ae. albopictus (1)	[1.702]			
Ae. albopictus	50%Human/50% Dog	*An. gambiae*-Human–Dog (21)	[1.686–2.006]	3	*Ae. albopictus*-Human–Dog (20)	[1.945–2.428]
		An. gambiae-Human–Dog–Sheep (2)	[1.746–1.853]			
Ae. albopictus	25%Human/75% Dog	*An. gambiae*-Human–Dog (13)	[1.736–2.269]	2	*Ae. albopictus*-Human–Dog (21)	[2.067–2.724]
		An. gambiae-Human–Dog–Sheep (5)	[1.899–2.136]			
		Ae. albopictus-Human(5)	[2.039–2.156]			
An. coluzzii	75%Human/25% Dog	*Ae .albopictus*-Human–Dog (4)	[1.739–1.929]	1	*An. coluzzii*-Human–Dog(9)	[1.948–2.932]
		Ae. albopictus-Human(5)	[1.927–2.345]			
		An. coluzzii-Human (1)	[2.027]			
An. coluzzii	50%Human/50% Dog	*Ae. albopictus*-Human–Dog (4)	[1.810–1.964]	1	*An. coluzzii*-Human–Dog(9)	[1.928–2.722]
		An. coluzzii-Human(4)	[1.876–2.264]			
		An. gambiae-Human(2)	[2.036–2.215]			
An. coluzzii	25%Human/75% Dog	*Ae. albopictus*-Human–Dog (8)	[1.722–2.216]	1	*An. coluzzii*-Human–Dog(9)	[2.111–2.835]
		An. gambiae-Human–Dog–Sheep(2)	[1.860–1.904]			
Total		99	51.51%	10	89	100%

(x)Number of specimens used for blind test analysis.
DB, database.

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Table 6. Influence of mosquito species on MALDI-TOF MS identification of *Aedes albopictus* and *Anopheles coluzzii* fed on human/sheep mixed blood meals

Mosquito species	Concentration of blood meal	Results of MS identification before upgrade (DB 1)	LSVs obtained before upgrade	Number of specimens used for DB upgrade	Results of MS identification after upgrade (DB 2)	LSVs obtained after upgrade
Ae. albopictus	75%Human/25% Sheep	*An. gambiae*-Human–Sheep (18)	[1.716–1.991]	2	*Ae. albopictus*-Human–Sheep(20)	[2.197–2.482]
		Ae. albopictus-Human (1)	[1.68]			
		An. gambiae-Human–Dog–Sheep(3)	[1.837–1.864]			
Ae. albopictus	50%Human/50% Sheep	*An. gambiae*-Human–Sheep (22)	[1.96–2.4]	3	*Ae. albopictus*-Human–Sheep(20)	[2.285–2.445]
		An. gambiae-Sheep (1)	[1.922]			
Ae. albopictus	25%Human/75% Sheep	*An. gambiae*-Human–Sheep (1)	[1.975]	2	*Ae. albopictus*-Human–Sheep(21)	[2.323—2.567]
		An. coluzzii-Sheep (20)	[1.637–1.958]			
		Ae. albopictus-cow (1)	[2.319]			
		An. coluzzii-mouflon(1)	[1.707]			
An. coluzzii	75%Human/25% Sheep	*An. coluzzii*-Human(1)	[2.096]	2	*An. coluzzii*-Human–Sheep (8)	[2.134–3.000]
		Aedes albopictus-Human(7)	[2.020–2.272]			
		Aedes albopictus-Cow(2)	[2.168–2.193]			
An. coluzzii	50%Human/50% Sheep	*An. gambiae*-Human–Sheep (3)	[1.855–1.969]	2	*An. coluzzii*-Human–Sheep (12)	[2.312–2.608]
		Ae. albopictus-Human–Sheep(7)	[1.805–1.993]			
		An. coluzzii-Sheep(1)	[1.826]			
		Ae. albopictus-Sheep(2)	[2.084–2.088]			
		An. gambiae-Human–Dog–Sheep(1)	[2.048]			
An. coluzzii	25%Human/75% Sheep	*Ae. albopictus*-Human–Sheep(3)	[1.736–1.748]	2	*An. coluzzii*-Human–Sheep (11)	[2.222–2.475]
		An. coluzzii-Sheep(4)	[1.689–1.932]			
		An. coluzzii-Mouflon(2)	[1.714–2.084]			
		Ae. albopictus-Sheep(3)	[1.770–2.215]			
		Ae. albopictus-Cow (1)	[2.146]			
Total		105	51.42%	13	92	100%

[(x)]Number of specimens used for blind test analysis.

This is considered significant in terms of influencing the transmission of pathogens and maintaining residual infectious diseases. For the identification of mixed blood meals using a proteomic approach, Önder *et al.* developed an experimental design using blacklegged ticks (*Ixodes scapularis*) (Onder *et al.*, 2013). Their approach successfully identified mixed blood meals from two vertebrates, depending on the concentration ratios. As for the identification of mixed bacterial species, Lin Zhang *et al.* reported that the major peaks in multi-bacterial samples could be assigned to those found in the respective spectra of each bacterium (Zhang *et al.*, 2015).

In our study, the last animal blood meal was correctly and unambiguously identified, although mosquitoes had firstly fed on human blood. This may be due to the interval of days (three) between the first and second blood meals of mosquitoes. The first blood meal was indeed digested by the mosquitoes. We obtained accurate identification with high LSVs (100%>1.8). This threshold is congruent with previously published studies on the use of MALDI-TOF MS to identify arthropods and their blood meals (Yssouf *et al.*, 2016). Interestingly, for closely related mosquito species, such as *An. gambiae* Giles and *An. coluzzii*, the mosquito species had little influence on blood meal identification, especially for successive blood meals. Indeed, with the exception of chicken blood, all *An. coluzzii* mosquito blood meals were correctly identified using *An. gambiae* Giles reference spectra. This was, however, not the case for *Ae. albopictus*, for which the identification of the last blood meal was more random if the reference spectra of the engorged abdomen of the corresponding species were not present in the database. These data suggest that mosquito proteins can have an impact on the identification of the blood meal, although the impact appears to be low between species belonging to closely related genera.

For mixed blood meals, the spectra from *Ae. albopictus* and even *An. coluzzii* fed with human/dog and human/sheep blood mixtures, respectively, revealed 51.51% and 51.42% correct identification if the database only contains the reference spectra of the engorged abdomen of *An. gambiae* mosquitoes. This could be explained by the diversity of reference spectra needed for accurate identification of mixed blood meal identification. Greater identification was obtained for *Aedes* and *An. coluzzii* mosquitoes when reference spectra corresponding to several blood mix ratios were available in the database (data not

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Table 7. Influence of mosquito species on MALDI-TOF MS identification of *Aedes albopictus* and *Anopheles coluzzii* triple blood meals

Mosquito species	Concentration of blood meal	Results of MS identification before upgrade (DB 1)	LSVs obtained before upgrade	Number of specimens used for DB upgrade	Results of MS identification after upgrade (DB 2)	LSVs obtained after upgrade
Ae. albopictus	Human/Dog/Sheep Equal concentration	*An. gambiae*-Human–Dog–Sheep (2)	[1.85–2.301]	3	*Ae. albopictus*-Human–Dog–Sheep(20)	[2.070–2.421]
		An. gambiae-Human–Sheep (21)	[1.875–2.49]			
An. coluzzii	Human/Dog/Sheep Equal concentration	*An. gambiae*-Human–Dog–Sheep (10)	[1.743–2.303]	1	*An. coluzzii*-Human–Dog–Sheep (9)	[2.391–2.725]
Total		33	36.36%	4	29	100%

(x)Number of specimens used for blind test analysis.
DB, database.

shown). The only reference spectra available for *An. gambiae* mixed blood meals were obtained from mosquitoes which were fed 50/50 blood mixtures. This bias could explain the low percentages of identification for mixed blood meals. This probably means that a larger database may be needed to identify blood meals from several mosquito species. However, an increasing number of laboratories (Raharimalala *et al.*, 2017; Halada *et al.*, 2018; Mewara *et al.*, 2018; Arfuso *et al.*, 2019; Chavy *et al.*, 2019; Rothen *et al.*, 2016; Sambou *et al.*, 2015; Yssouf *et al.*, 2016) are applying MALDI-TOF to MS medical entomology and Raharimalala *et al.* have also highlighted the need for sharing and have expressed interest in an international database (Raharimalala *et al.*, 2017). Many reference spectra are also available on request or directly online (Lawrence *et al.*, 2019), substantially facilitating database creation. The MALDI-TOF mass spectrometry approach may also appear to be limited by the cost of the device but when the MALDI-TOF MS device is bought for clinical microbiology purposes, it can also be used for medical entomology at no additional cost. For example, in Senegal, Masse *et al.* have successfully identified the *Culicoides* spp. using protein-based MALDI-TOF MS, which is economically advantageous compared to molecular tools, some sequences of which are not available in Genbank (Sambou *et al.*, 2015).

ELISA is a widely used approach that can be reliable for identifying frequently encountered hosts but much more challenging for wild hosts for which enzyme-labelled antibodies are not yet commercially available (Beier *et al.*, 1988). In previous studies on Culicidae, up to 35% of the blood meals could not be identified using ELISA or were attributed to an unidentified mammal or bird (Apperson *et al.*, 2002). As for molecular approaches, DNA prepared from the engorged abdomen not only can be used for blood meal identification but also can be used for arthropod identification or microorganism detection by PCR (Maleki-Ravasan *et al.*, 2009).

The major advantages of using MALDI-TOF MS are its rapidity, effectiveness and reliability in identifying successive blood meals and mixed blood meals. In this study, only a small quantity of host blood was used for MALDI-TOF MS analysis, and the remaining material can be used for other studies, including the legs to identify mosquitoes at the species level (Yssouf *et al.*, 2013; Nebbak *et al.*, 2016).

In this study, we concluded that the species of mosquito has very little impact on blood meal identification if they are closely related. *Anopheles coluzzi* and *An. gambiae* Giles belong to the same complex, and are therefore not representative of the diversity of the *Anopheles* genus. More species of this genus should be included to draw conclusions regarding the impact of mosquito species on blood meal identification. Furthermore, as illustrated with *Ae. albopictus* mosquitoes in this study, identification of blood meals using a database created with a very different mosquito species can be unreliable. Nevertheless, the databases can be easily shared in order to circumvent this drawback.

Under an epidemiological perspective for the transmission of human pathogens, mosquito vectors should feed on a person or an animal and subsequently, on other human beings. In this preliminary study, we used a first human blood meal and the second blood meal on different vertebrate species (mostly domestic animals). These hosts were selected as some may act as reservoirs for zoonotic pathogens, and were available in our laboratory. To date, the performance of MALDI-TOF MS has not been determined yet for the distinction of human bloods, challenging the interpretation of MS identification of successive or mixed human blood meals.

Conclusion

Herein this paper, we provide more data to support the fact that MALDI-TOF MS appears as a rapid, reliable and cost-effective method for identifying *An. gambiae* Giles, *An. coluzzii* and *Ae. albopictus* blood meals. Correct identification was observed in all samples tested after the database was upgraded. Although MS profiling has been shown to be effective in this regard, our technique still needs to be tested on specimens collected in the field where feeding parameters are less controlled than in laboratory conditions. All spectra of the arthropod spectra database are available on request for other collaborative research teams.

Conflict of interest

The authors declare that they have no competing interests.

Ethical standards

Human blood was obtained from the national French blood bank, the 'Etablissement Français du Sang' (EFS) accredited by the human and animal ethics committees of the Institutional Animal Care Committee of IHU Méditerranée Infection. Consents for blood donation were collected at EFS. Animal studies were conducted in line with Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010, and in compliance with French Government Decree No. 2013-118 of 1 February 2013. The study was approved by the local Ethics Committee (Comité d'Ethique en Expérimentation Animale, Marseille) and the Institutional Animal Care Committee in Marseille, France.

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Availability of data and materials

All datasets regarding this study are included in the main paper and the additional supporting files.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S003118201900163X

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Author contributions. AL, PP and ML designed the experiments. FT and SN performed the experiments. FT, SN, ML and LA analysed the data. PP, OKD, BD and DR provided reagents/materials/analysis tools. FT, ML and NS wrote the paper. ML and PP coordinated the writing of the paper. All authors reviewed the final version.

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