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Research

Identification of Lice Stored in Alcohol Using MALDI-TOF MS

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Abstract

Lice pose major public and veterinary health problems with economic consequences. Their identification is essential and requires the development of an innovative strategy. MALDI-TOF MS has recently been proposed as a quick, inexpensive, and accurate tool for the identification of arthropods. Alcohol is one of the most frequently used storage methods and makes it possible to store samples for long periods at room temperature. Several recent studies have reported that alcohol alters protein profiles resulting from MS analysis. After preliminary studies on frozen lice, the purpose of this research was to evaluate the influence of alcohol preservation on the accuracy of lice identification by MALDI-TOF MS. To this end, lice stored in alcohol for variable periods were submitted for MS analysis and sample preparation protocols were optimized. The reproducibility and specificity of the MS spectra obtained on both these arthropod families allowed us to implement the reference MS spectra database (DB) with protein profiles of seven lice species stored in alcohol. Blind tests revealed a correct identification of 93.9% of *Pediculus humanus corporis* (Linnaeus, 1758) and 98.4% of the other lice species collected in the field. This study demonstrated that MALDI-TOF MS could be successfully used for the identification of lice stored in alcohol for different lengths of time.

Key words: lice, MALDI-TOF MS, storage method, identification

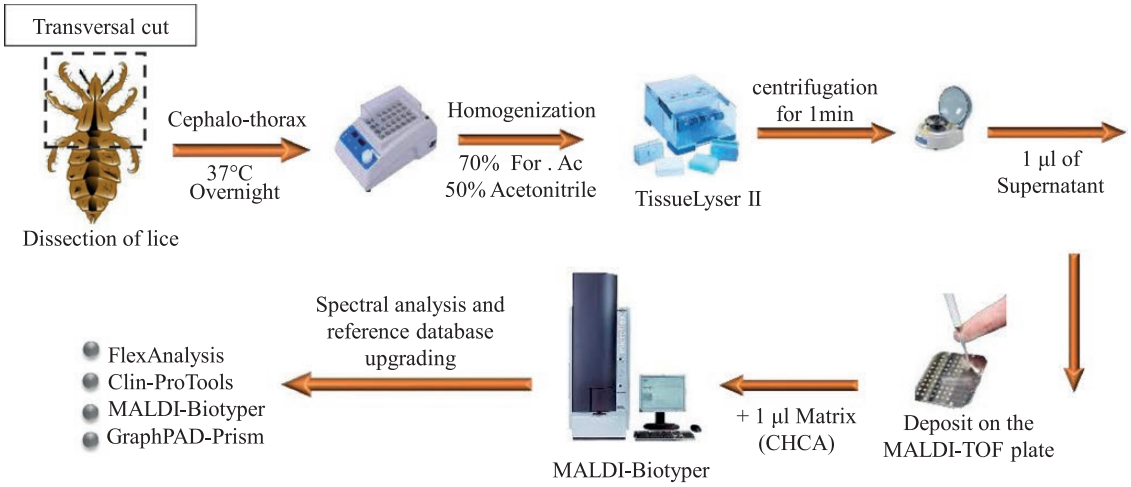
Résumé

Les poux posent des problèmes majeurs de santé publique et vétérinaire, avec des conséquences économiques. Leur identification est essentielle et nécessite le développement d'une stratégie innovante. Le MALDI-TOF MS a récemment été proposé comme un outil rapide, peu coûteux et précis pour l'identification des arthropodes. L'alcool est l'une des méthodes de stockage les plus fréquemment utilisées et permet de conserver les échantillons pendant de longues périodes à température ambiante. Plusieurs études récentes ont rapporté que l'alcool modifie les profils protéiques résultant de l'analyse de la SEP. Après des études préliminaires sur les poux congelés, le but de cette recherche était d'évaluer l'influence de la conservation de l'alcool sur la précision de l'identification des poux par la SEP MALDI-TOF. À cette fin, des poux conservés dans l'alcool pendant des périodes variables ont été soumis à l'analyse de la SEP et les protocoles de préparation des échantillons ont été optimisés. La reproductibilité et la spécificité des spectres de MS obtenus sur ces arthropodes nous ont permis de mettre en œuvre la base de données des spectres de MS de référence (DB) avec les profils protéiques de sept espèces de poux stockés dans l'alcool. Des tests en aveugle ont révélé une identification correcte de 93,9% de *Pediculus humanus corporis* (Linnaeus, 1758) et de 98,4% des autres espèces de poux collectées sur le terrain. Cette étude a démontré que le MALDI-TOF MS pouvait être utilisé avec succès pour l'identification des poux stockés dans l'alcool pendant différentes durées.

Mots-clés: Poux, MALDI-TOF MS, méthode de stockage, identification.

Graphical abstract

Protocol established for MALDI-TOF MS analysis of lice in alcohol 70%.



Lice are apterous insects that belong to theorder of Phtiraptera. They are mandatory ectoparasites of mammals and birds. More than 4,900 species of lice have been described in the literature (Johnson et al. 2004). Lice are separated into two suborders: Anoplura (sucking lice) and Mallophaga (chewing lice). Two genera are recognized within the human sucking lice order (Phthiraptera: Anoplura), *Pthirus* and *Pediculus*. Each genus is presented by one species, *Phtirius pubis* (Linnaeus, 1758) and *Pediculus humanus* (Linnaeus, 1758) (Louni et al. 2018, Amanzougaghene et al. 2020). Both louse species are obligate blood-feeding parasites that have gorged on human blood for thousands of years (Amanzougaghene et al. 2020). The sucking lice feed exclusively on the blood of mammals (Boyd and Reed 2012). Chewing lice infest birds, mammals and feed mainly on epidermal debris from their hosts (Shao et al. 2015).

Lice such as the body louse (*Pediculus humanus humanus*) and head louse (*Pediculus humanus capitis*) (Linnaeus, 1758), which live in clothing and on the human scalp, respectively, are ectoparasites of great concern to public health (Louni et al. 2018). Lice have also been reported to be a veterinary problem, inducing economic loss, notably for livestock (Titchener 1985). These consequences can be summarized in some countries around the world by blood spoliation, anemia, stress, hair loss, severe pruritus, decreased appetite and milk production, focal necrosis, and scarring on the skin of heavily infested animals (Domínguez-Peñafiel et al. 2011, Álvarez-Ortega and Peña-Santiago 2013). Body lice are very common in people living in unstable and impoverished conditions (Brouqui 2011), unlike head lice, which infest school children, regardless of the level of hygiene (Izri et al. 2010). Outside of their biotopes, the two species of lice are morphologically indistinguishable (Drali et al. 2013). Body lice transmitted infections form are part of the epidemics that have been described during wars throughout history (Raoult and Roux 1999). They are known to be vectors of human disease including *Bartonella quintana*, the agent of trench fever, *Borrelia recurrentis*, the agent of louse-borne relapsing fever, *Rickettsia prowazekii*, the agent of epidemic typhus (Raoult et al. 1998, Amanzougaghene et al. 2020), *Yersinia pestis*, the causal agent of plague and are also responsible for phtiriosis, otherwise known as pediculosis (McNair 2015). The potential of head lice as a disease vector has not yet been clearly established, despite the detection of the DNA of several pathogens in head lice (Mana et al. 2017, Amanzougaghene et al. 2020).

Morphological identification requires entomological expertise and accurate identification is sometimes not possible, notably for damaged or immature specimens, due to the lack of crucial criteria or identification keys (Dupuis et al. 2018). To overcome these drawbacks, molecular approaches, such as sequencing the *18S*rRNA gene, have been developed for lice identification (Whiting 2002). The sequencing of cytochrome c oxidase subunit I (*COI*) gene of lice has also been used for their identification (Johnson et al. 2003). Recently, matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF/MS) has been evaluated as a rapid tool for the identification of arthropods, including ticks (Boyer et al. 2017), mosquitoes (Vega-Rúa et al. 2018), culicoides (Sambou et al. 2015), and phlebotomine sand flies (Lafri et al. 2016). MALDI-TOF MS has also been successfully applied for the identification of frozen lice (Ouarti et al. 2020). The large majority of the arthropod families submitted to MALDI-TOF MS for identification have been performed on fresh or frozen specimens (Yssouf et al. 2016). However, arthropod collection sites are often far from laboratories, which require the use of storage methods other than freezing, methods which are less restrictive for sample preservation. Alcohol at 70% or 90% (v/v) is one of the storage methods frequently used, preserving samples for long periods at room temperature (Song et al. 2016). Recent studies have reported a modification of MS profiles between fresh or frozen specimens compared to counter specimens preserved in alcohol buffer (Nebbak et al. 2016, 2017). However, the stability over time of MS profiles from some tick and flea species stored in alcohol underline that MALDI-TOF MS could also be used for the identification of arthropods stored in this buffer (Diarra et al. 2017, Zurita et al. 2019).

The purpose of this study was to assess the influence of alcohol preservation on the accuracy of identification of lice by MALDI-TOF MS. For this purpose, one laboratory-reared louse species, *P. humanus corporis*, was preserved in alcohol for periods ranging from 2 mo to 1 yr to assess the efficacy of MALDI-TOF MS for the correct identification of these arthropods. In addition, seven lice species from field, stored in alcohol from between a few months to

several years, and which had been morphologically identified, were submitted to MALDI-TOF MS to assess the intraspecies reproducibility and interspecies specificity of resulting MS spectra.

Materials and Methods

Arthropod Rearing and Storing

Adult *P. humanus corporis* lice were reared in a climatic chamber (25°C, relative humidity 80–90%) and successive generations were obtained by allowing the lice to feed on rabbits, as previously described (Mana et al. 2017). Two hundred adult specimens were sedated at –20°C prior to being stored in 70% (v/v) alcohol at room temperature. Fifteen specimens stored in alcohol were collected every month for MS analysis between months 2 and 12.

Field Collection of Arthropods

Seven lice species (*Bovicola caprae* (Gurlt, 1843), *Goniocotes gallinae* (Geer, 1778), *Goniodes gigas* (Taschenberg, 1879), *Goniodes dissimilis* (Denny, 1842), *Haematopinus eurysternus* (Nitzsch, 1818), *Menacanthus stramineus* (Nitzsch, 1818), and *Menopon gallinae* (Linnaeus, 1758), collected from animals from the 'El-Taref' area (Algeria) were identified using morphological keys (Meguini et al. 2018). Between 9 and 15 specimens per species were included, representing a total of 75 specimens. All lice collected were immediately stored in 70% (v/v) alcohol prior to morphological identification and being sorting by species. Details about number of specimens per species, storage duration, and mammal host origin are available in Table 1.

Sample Preparation for MALDI-TOF Analysis

The cephalo-thorax for some specimens was retained for MALDI-TOF MS tests (Yssouf et al. 2013). The abdomens of each specimen were frozen at –20°C for later molecular biology analysis. The cephalo-thorax was placed in an oven at 37°C overnight, to evaporate organic solvents (Diarra et al. 2017). The cephalo-thorax of each louse specimen were then homogenized using TissueLyser (Qiagen) with a pinch of glass powder (Sigma, Lyon, France) as disruptor. The parameters selected for sample homogenization were six cycles of 60 s at a frequency of 30 Hertz. Then microliters of homogenization buffer were used for the lice conserved in alcohol (70%v/v). The homogenization buffer was composed of a 50/50 (v/v) mix of formic acid (70%v/v) (Sigma) plus acetonitrile (50% v/v) (Fluka, Buchs, Switzerland). Two fresh *P. humanus corporis* specimens were homogenized using the same automated conditions, and used as positive controls.

Molecular Identification of Lice

The two specimens per species definitely identified by morphological criteria and selected for MS DB creation, were selected (Table 1). The abdomen was used for DNA extraction using a Qiagen kit (Qiamp DNA mini kit, Hildesheim, Germany) according to the manufacturer's instructions, after mechanical homogenization. Species-level molecular identification attempts of the lice were performed by sequencing a fragment of the 18SrRNA gene (Ouarti et al. 2020). Primers SAIDG: (5′-TCTGGTTGATCCTGCCAGTA-3′) and SBIDG: (5′-ATTCCGATTGCAGAGCCTCG-3′) were used to amplify the 18SrRNA gene and LCO1490: 5′-GGTCAACAAATCAT AAAGATATTGG-3′; HC02198: 5′-TAAACTTCAGGGTGACCAA AAAATCA-3′) (Folmer et al. 1994) for molecular identification of lice in this study. Abdomens of two *P. humanus corporis* specimens freshly collected or stored in alcohol during 6–12 mo were used as extraction and PCR controls.

Sample Loading on MALDI-TOF Target Plates

After sample homogenization, centrifugation at 200 g was performed for 1 min to pellet debris, and 1 µl of supernatant from each sample was loaded on the MALDI-TOF MS target plate in quadruplicate (Bruker Daltonics, Wissembourg, France) and covered with 1 µl of CHCA matrix solution, composed of saturated alpha-cyano-4-hydroxycynnamic acid (Sigma, Lyon, France), 50% acetonitrile (v/v), 2.5% trifluoroacetic acid (v/v) (Aldrich, Dorset, UK), and HPLC-grade water. After drying for several minutes at room temperature, the target was placed in the Microflex LT MALDI-TOF Mass Spectrometer (Bruker Daltonics) for analysis. To control the matrix quality, sample loading, and MALDI-TOF apparatus performance, the matrix solution was loaded in duplicate on to each MALDI-TOF plate, with and without two fresh lice prepared under the same conditions, which served as grinding controls.

MALDI-TOF MS Parameters

Protein mass profiles were generated using a Microflex LT MALDI-TOF Mass Spectrometer (Bruker Daltonics, Germany), 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 Auto Xecute method of the flex Control v2.4 software (Bruker Daltonics). The spectrum profiles were visualized with flex Analysis v3.3 software and were exported to ClinPro Tools v2.2 and MALDI-Biotyper v3.0 (Bruker Daltonics, Germany) for data processing (smoothing,

Table 1. Overview of lice origins, subgroup selected for MALDI-TOF MS reference database creation a priori blind tests for MS identification and Log-score values for each species

Species	Host	Storage time (years)[a]	Total number of specimens	Number of specimens included in the database and sequenced	Species identified by MALDI-TOF MS	LSVs
Bovicola caprae	Goats	(2014) 5	10	2	*B. caprae*	[2.18–2.43]
Goniocotes gallinae	Poultry	(2014) 5	9	2	*G. gallinae*	[2.01–2.25]
Goniodes gigas	Poultry	(2015) 4	9	2	*G. gigas*	[2.03–2.35]
Goniodes dissimilis	Poultry	(2015) 4	15	2	*G. dissimilis*	[1.77–2.30]
Haematopinus eurysternus	Cattle	(2014) 5	11	2	*H. eurysternus*	[2.00–2.24]
Menacanthus stramineus	Mammals	(2015) 4	10	2	*M. stramineus*	[1.82–2.13]
Menopon gallinae	Poultry	(2016) 3	11	2	*M. gallinae*	[1.91–2.39]
Total			75	14		

[a]All specimens were stored in 70% (v/v) alcohol.

baseline subtraction, and peak picking) and evaluation with cluster analysis.

Spectral Analysis and Reference Database Upgrading

Intraspecies reproducibility and interspecies specificity of MS spectra were visually compared using the average spectra (main spectrum profile [MSP]) obtained from the four spectra of each sample tested using the flex Analysis v3.3 and ClinPro Tools v2.2 software (Bruker Daltonics). To create a database specific to lice samples preserved in alcohol, reference spectra (MSP) were created by combining the spectra replicate results from each selected specimen using the automated function of the MALDI-Biotyper software v3.0. (Bruker Daltonics). At least two specimens per species were used. MSPs were created on the basis of an unbiased algorithm using information on the peak position, intensity and frequency data. For *P. humanus corporis*, MS spectra from two specimens that had been freshly collected or stored in alcohol for 2, 4, and 6 mo were selected to create reference MS spectra. A total of eight *P. humanus corporis* specimens were then included in the database. Concerning the seven lice species collected in the field and stored in alcohol, MS spectra from two specimens per species were included in the reference MS spectra database, representing a total of 14 specimens. These 14 specimens were subjected to standard PCR using 18SrRNA and mCOI genes as previously described (Folmer et al. 1994, Ouarti et al. 2020). The MS spectra from the specimens included in the database were used for clustering analysis and to increment our homemade database using MALDI-Biotyper v.3.0. Software (Bruker Daltonics, Germany). Cluster analyses (MSP dendrogram) were performed to determine how organisms are related to one another. The setting parameters were as follows: distance measure by correlation, linkage by average, the score threshold value for a single organism was 300 (arbitrary unit), and for related organisms was 0 (arbitrary unit).

Blind Test for Study Validation

Blind tests were performed with new louse specimens which were either laboratory reared or collected in the field. Total spectra of 226 specimens encompassing eight lice species were tested against the upgraded homemade MS reference spectra database. The level of identification significance was determined using the log score values (LSVs) given by the MALDI-Biotyper software v.3.3, corresponding to a matched degree of signal intensities of mass spectra of the query and the reference spectra. LSVs ranging from 0 to 3 were obtained for each spectrum of the samples tested. Based on previous studies (Yssouf et al. 2013, Dieme et al. 2014), an LSV greater than 1.8 was considered as the cut-off for species identification. Data were analyzed using GraphPad Prism software version 5.01 (GraphPad, San Diego, CA).

Results

Assessment of Lice Preservation Modes and Duration Compatibilities With MALDI-TOF MS Analyses

Fifteen lice specimens from *P. humanus corporis* species, stored in alcohol from between 2 and 12 mo were submitted for MS analysis. MS spectra from fresh *P. humanus corporis* specimens were particularly different from those of counter species stored in alcohol (Fig. 1(i)). MS spectra from lice stored for the same length of time in alcohol and between different time points presented visually similar MS spectra. To verify the reproducibility of MS spectra whatever the length of time they spent in alcohol, an MSP dendrogram was performed using two specimens per storage condition and duration (Fig. 1(ii)). Specimens stored in alcohol were grouped in a distinct branch from those freshly submitted to MS from the same species (i.e., *P. humanus corporis*). Moreover, no ordination of the MS spectra was noted according to the length of time the sample was stored in alcohol. The mixing of the samples revealed that these MS profiles appeared to be stable, independently of the length of time they were stored in the alcohol buffer.

To confirm these results, MS spectra from 15 specimens per time point were queried against the homemade MS spectra reference database upgraded with MS profiles from six *P. humanus corporis* samples stored in alcohol for 2, 4, and 6 mo (two specimens per time point). Of the 165 MS spectra queried against the database, 97.6% (n = 161/165) of them were correctly identified at species level, matching with MS spectra from *P. humanus corporis* stored in alcohol (Fig. 1(iii)). The four MS spectra which were not identified came from specimens stored for three and 10 mo, and were of very low quality (low MS peak intensity and diversity), explaining why they were not correctly identified (LSVs < 1.2). The LSVs of the remaining samples which were correctly identified (n = 161) ranged from 1.34 to 2.65. To consider the identification as reliable, LSVs should reach the threshold of 1.8. Here, 93.9% (n = 155/165) of MS spectra succeeded in reaching this threshold (LSVs > 1.8). Although the LSV threshold of 1.8 is considered for identification relevant of arthropods, for microorganism identification LSVs upper or equal to 1.7 and 2.0 were established for genus and species identification, respectively. The application of these thresholds revealed that 96.4% (159/165) of *P. humanus corporis* were identified at least at the genus level and 63.0% (104/165) at the species level. It is interesting to note that high LSVs were also obtained for MS spectra corresponding to specimens stored in alcohol for which respective time points were not included in the database. These results underlined the reproducibility of the MS spectra between these specimens, independently of the length of time the specimens were preserved in ethanol.

MS Database Creation and Blind Tests for Lice Identification by MALDI-TOF MS

To assess whether species-specific MS profiles could be obtained for lice preserved in alcohol for long periods, 75 specimens from seven lice species stored in alcohol for between 3 and 5 yr, were included in this study (Table 1). A subgroup of 14 lice which had been morphologically identified, including two specimens per species, were selected for evaluating intraspecies reproducibility and interspecies specificity of MS spectra (Table 1). To confirm their morphological identification, molecular assays were done. Unfortunately, neither 18SrRNA nor mCOI target gene sequences were successfully amplified using DNA extract from 14 lice stored long time in alcohol. Conversely, PCR products were obtained from DNA extracts of *P. humanus corporis* specimens freshly collected or stored in alcohol during 6–12 mo, for both gene targets. The sequencing and BLAST of these gene products matched at 99% of identity and 100% coverage with *P. humanus corporis* (18S RNA: FJ267398.1 and COI: KP143370). A visual analysis of the MS profiles from the cephalothorax of lice using the Flex Analysis software indicated that they seem to be reproducible between specimens from the same species (Fig. 2(i)). Moreover, the MS profiles appeared to be distinct between lice species. The intraspecies reproducibility and interspecies specificity of MS profiles was objectified by cluster analysis. Cluster

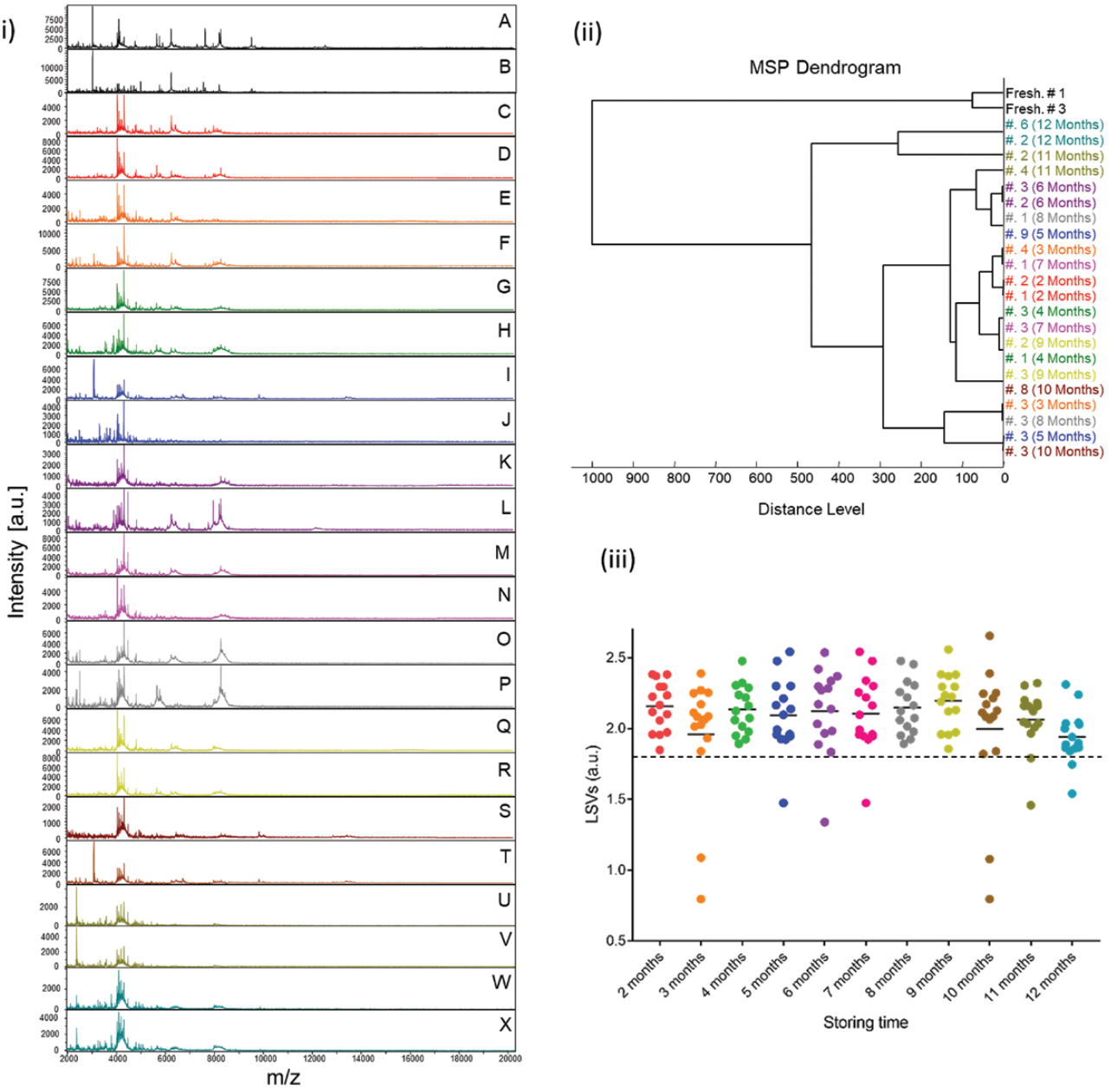


Fig. 1. Consequences of storage mode and duration on mass spectrometry (MS) spectra of lice cephalo-thorax and their reliable identification. (i) Representative MS spectra of cephalo-thorax of adult *Pediculus humanus corporis* fresh (A, B) or stored for 2 (C, D), 3 (E, F), 4 (G, H), 5 (I, J), 6 (K, L), 7 (M, N), 8 (O, P), 9 (Q, R), 10 (S,T), 11 (U,V), or 12 (W, X) months in alcohol 70% v/v. (ii) Reproducibility and specificity of MALDI-TOF MS spectra from *P. humanus corporis* lice.Two specimens per storing mode (fresh vs alcohol) and time of storing (from 2 to 12 mo in alcohol) were used to construct the MSP dendrogram. #: specimens number. The dendrogram was created using Biotyper v3.0 software and distance units correspond to the relative similarity of MS spectra. (iii) Comparison of LSVs obtained for 15 *P. humanus corporis* specimens stored in alcohol tested monthly against the upgraded homemade MS reference database. Dashed line represents the threshold value for reliable identification (LSV > 1.8). a.u., arbitrary units; m/z, mass-to-charge ratio.

analysis revealed clustering around distinct branches of lice according to species (Fig. 2(ii)). However, lice from the same genus (e.g., *Goniodes*) were not all clustered on the same branch of the MSP dendrogram. Interestingly, MS spectra from *P. humanus corporis*, stored in alcohol for 6 mo, were separated on the MSP dendrogram from the other lice species, underlining the specificity of their MS spectra. Based on these results, the spectra of these 14 specimens from these seven new species were used to increment the homemade MS reference spectra database. The 61 remaining lice were submitted for MS analysis. Resulting MS profiles were then used to query the homemade MS reference spectra database (Fig. 2(iii)). Overall, the query against the upgraded MS reference spectra database revealed LSVs ranging from 1.77 to 2.43 with 100% classification, corroborating the morphological classification. An LSV threshold for considering an identification as relevant was established at 1.8 (Yssouf et al. 2013), and only one *G. dissimilis* specimen did not reach this threshold and could not be validated. The rate of relevant identification was ultimately established at 98.4% (n = 60/61). If the thresholds established for micro-organisms identification were taken into account for the lice, 100% (61/61) and 78.69% (48/61) reached the 1.7 and 2.0 cut-off LSVs corresponding to genus and species classification, respectively.

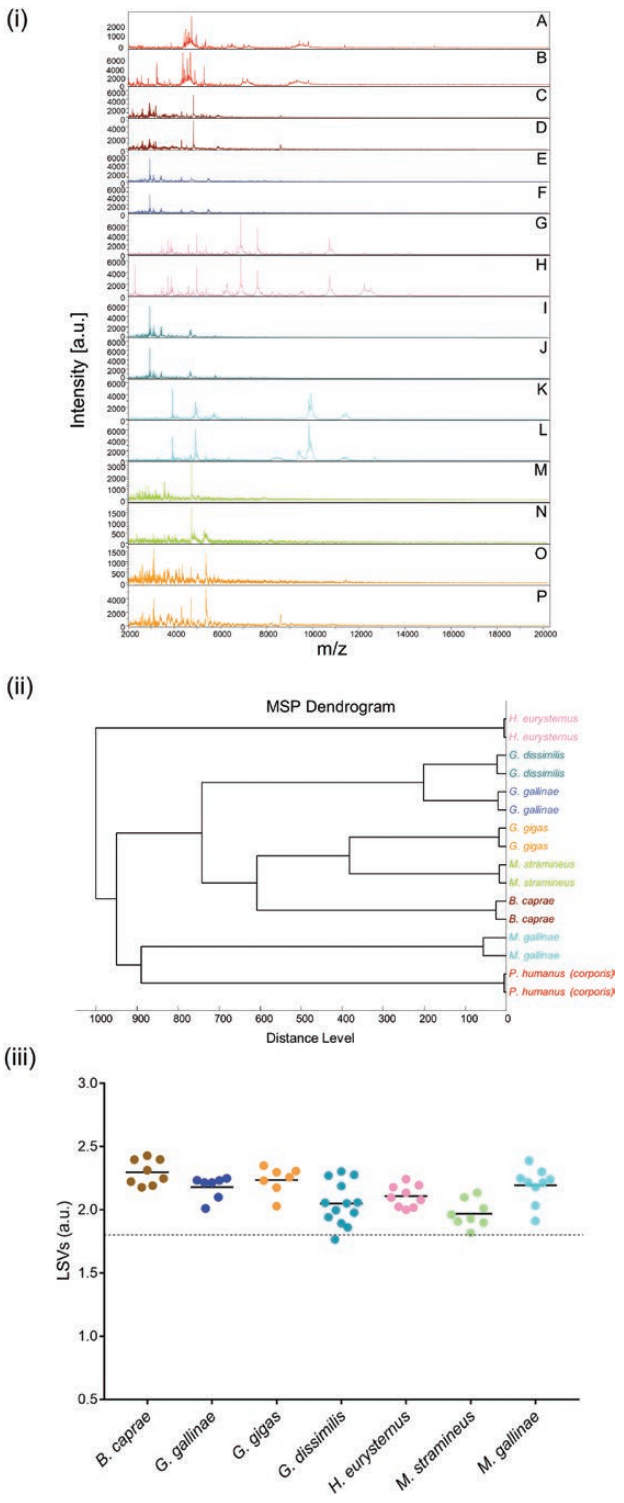


Fig. 2. Specific MALDI-TOF MS spectra of eigth lice species stored in alcohol and assessment of their MS identification. (i) Representative lice MS spectra of cephalo-thorax from *P. humanus corporis* (A, B), *B. caprae* (C, D), *G. gallinae* (E, F), *H. eurysternus* (G, H), *G. dissimilis* (I, J), *M. gallinae* (K, L), *M. stramineus* (M, N), and *G. gigas* (O, P) stored in alcohol. (ii) MSP dendrogram constructed using two representative MS spectra from the eight distinct lice species stored in alcohol. (iii) Comparison of LSVs obtained for 7 to 13 specimens per lice species stored in alcohol against the upgraded homemade MS reference database with MS spectra from respective species stored in alcohol. Dashed lines represent the threshold value for reliable identification (LSV > 1.8). a.u., arbitrary units; m/z, mass-to-charge ratio.

Discussion

For several years, the MALDI-TOF MS technique has been established for the routine diagnosis of micro-organisms such as the identification of bacteria (Seng et al. 2009, Bizzini and Greub 2010), archaea (Dridi et al. 2012), yeasts, and filamentous fungi (Seng et al. 2009, Dridi et al. 2012). This innovative proteomic tool was also successfully applied for the detection of other pathogens such as *helminths* and intestinal *protozoa* of medical and veterinary importance (Feucherolles et al. 2019). Most of these studies were based primarily on the identification of specific proteins rather than entire pathogens. However, others works reported the use of MALDI-TOF MS for species-specific identification of nematode species, *Trichinella* spp. and *Entamoeba* spp. (Calderaro et al. 2015, Mayer-Scholl et al. 2016, Bredtmann et al. 2017). Since then, the MALDI-TOF MS process has been successfully tested in medical entomology to identify different arthropod families including culicoides biting midges (Dieme et al. 2014), mosquitoes (Müller et al. 2013, Yssouf et al. 2013, Yssouf et al. 2014), phlebotomines sand flies (Mathis et al. 2015, Lafri et al. 2016), fleas (Yssouf, et al. 2014), Tsetse flies (Hoppenheit et al. 2013, 2014), and ticks (Yssouf et al. 2013a). These studies concerned mainly fresh or frozen stored arthropods. However, due to the location of the place of collection (far from the laboratory, lack of infrastructure, and transport time), the resources available in the field mean that other means of sample conservation are required. One of the most frequently method used for arthropods is storage in alcohol (Zurita et al. 2019). Previous work has shown that storing arthropods in alcohol modifies MS profiles compared to fresh specimens of the same species, hampering direct identification by MALDI-TOF MS against the reference MS spectra database which only includes counter species from fresh or frozen specimens (Kumsa et al. 2016, Diarra et al. 2017).

This study revealed that the MALDI-TOF MS profiles of *P. humanus corporis* stored in alcohol were different from the fresh ones of the same species. The differences in MS profiles according to storage for specimens from the same species have already been reported (Diarra et al. 2017, Zurita et al. 2019). Nevertheless, despite the variation of MS profiles due to storage in alcohol, it was underlined that the MS spectra remained reproducible for specimens of the same species stored for varying periods in alcohol. The intraspecies stability of the MS spectra is a key factor for suitable specimen identification using the MALDI-TOF MS tool (Diarra et al. 2017). The inclusion of MS spectra from specimens stored in alcohol is therefore essential for the reliable identification of counter species stored in the same organic buffer (Nebbak et al. 2017).

This strategy was successfully applied to *Ixodid* ticks from Ethiopia which were conserved in alcohol 70% (v/v) (Kumsa et al. 2016). It was shown that, despite storing the ticks for a long time (2 yr or more), the MS spectra were reproducible for specimens of the same species. These results were confirmed more recently, underlining the reproducibility and stability of tick and flea MS spectra from specimens stored in alcohol for periods of time (Nebbak et al. 2017). In addition, the sample preparation for MS analysis was standardized, which should facilitate sharing and exchanging results between research teams (Nebbak et al. 2017). Here, the homogeneity and specificity of the MS spectra from specimens stored in alcohol were validated by the clustering and intertwining of the MS spectra from *P. humanus corporis* lice samples stored in 70% alcohol whatever the period of conservation compared to the fresh one. The query against the upgraded MS spectra reference database

with *P. humanus corporis* samples stored in alcohol enabled relevant identification (LSV > 1.8) for nearly 94% of them.

A recent study reported on the application of MALDI-TOF MS profiling for the identification of 13 lice species of veterinary importance (Ouarti et al. 2020). In this previous study, the cephalo thorax appeared as the best body part for MS submission. All these specimens, collected from three distinct Algerian sites, Souk-Ahras, Guelma, and El-Taref, were stored at –20°C prior to MS analysis. Although six lice species (*Bovicola caprae*, *Goniocotes gallinae*, *Goniodes gigas*, *Haematopinus eurysternus*, *Menacanthus stramineus*, and *Menopon gallinae*) were shared with the present study, the MS spectra are not super imposable due to the different storage method. Here, the conservation of the lice in 70% ethanol buffer generated modifications to MS profiles compared to fresh or frozen stored counterpart species, as observed for *P. humanus corporis*. This phenomenon was previously repeatedly reported for ticks (Diarra et al. 2017, Nebbak et al. 2017), culicoides (Sambou et al. 2015), and fleas (Zurita et al. 2019).

It is interesting to note that 18S RNA and COI target gene sequences were successfully amplified using DNA extracts from *P. humanus corporis* specimens freshly collected or stored in alcohol during 6–12 mo. The failing of gene amplifications in the samples from the seven other lice species was not attributed to impairments of DNA extract or PCR experiments but rather likely to an inappropriate storing of lice samples. All the lice were stored in 70% ethanol during 4–6 yr at room temperature. Previous works reported that the preservation of sample in 70% ethanol is less efficient than 96–100% ethanol notably concerning DNA integrity (Barnes et al. 2000, Doorenweerd and Beentjes 2012). Effectively, 70% ethanol could induce incomplete fixation of the sample responsible for the degradation of the DNA, but also it low quality could participate to DNA hydrolysis (Spigelman et al. 2001).

The body part selected for MS analysis was the cephalo-thorax, which presented reproducible and specific MS profiles. The volume of homogenization buffer was adjusted to optimize the quality of the MALDI-TOF MS profiles. The main limitations are the small size of the lice, generating low protein quantities and MS spectra of lower intensity, but also the difficulty of sample grinding which made it necessary to increase the number of homogenization cycles. Compared to the standardized protocols on ticks (Diarra et al. 2017) and mosquitoes (Yssouf et al. 2013b), the grinding time was doubled and the homogenization buffer was decreased. These modifications made it possible to obtain reproducible and specific profiles for lice but different from fresh counter species, as mentioned previously for other arthropod families (Yssouf et al. 2013a). Despite these optimizations, the MS profiles continue to be low in intensity. This low intensity is mainly linked to the small size of the cephalo-thorax of lice. MS profiles with low intensity had already been reported, for instance when the early developmental stages of mosquito larvae were analyzed by MALDI-TOF MS (Dieme et al. 2014), and confirmed in a larger study aiming to monitor mosquito larvae in Marseille (Nebbak et al. 2018). Nevertheless, in this study, repeatable and specific MS spectra were obtained between seven species of lice kept in alcohol for several years. The query of the upgraded MS reference database with MS profiles from lice species stored in alcohol showed that 100% of the specimens were relevantly identified, regardless of the length of time they were stored in alcohol, confirming the use of this proteomic tool for the identification of lice stored in alcohol. The intraspecies reproducibility of the MS spectra from specimens stored in alcohol is a key factor for their reliable identification with this proteomic tool. The preservation of lice in alcohol is less restrictive than the freezing method and also makes it easier to transport or transfer samples. The enrichment of the MS spectra DB in the near future with additional lice species stored in alcohol will be essential if this tool is to be used for lice identification.

This innovative tool is to propose to physicians, researchers, or lab technicians an alternative strategy to morphological identification which requires entomologist competence or to molecular assays for which the price remains relatively high compared to MS analysis for entomological studies. The high throughput, low cost, rapidity, and accuracy of this approach could become a new tool to monitor arthropod vectors collected in the field or on mammalian hosts including humans. A rapid identification of arthropods collected on human could be helpful for physician to propose preventive treatment of pathogenic agents. We are convinced of the high potential of the MALDI-TOF MS profiling for entomological diagnosis and more globally for medical entomology.

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