Effect of trapping method on species identification of phlebotomine sandflies by MALDI-TOF MS protein profiling

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Abstract. Sandflies (Diptera: Psychodidae) (Newstead, 1911) are blood-feeding insects that transmit human pathogens including Leishmania (Trypanosomatida: Trypanosomatidae) parasites, causative agents of the leishmaniases. To elucidate Leishmania transmission cycles, conclusive identification of vector species is essential. Molecular approaches including matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) protein profiling have recently emerged to complement morphological identification. The aim of this study was to evaluate the effect of the trap type used to collect sandflies, specifically Centers for Disease Control (CDC) light or sticky traps, the two most commonly used in sandfly surveys, on subsequent MALDI-TOF MS protein profiling. Specimens of five species (Phlebotomus ariasi, Phlebotomus papatasi, Phlebotomus perniciosus, Phlebotomus sergenti, Sergentomyia minuta) collected in periurban and agricultural habitats in southeast Spain were subjected to protein profiling. Acquired protein spectra were queried against an in-house reference database and their quality assessed to evaluate the trap type effect. The results indicate that trap choice can substantially affect the quality of protein spectra in collected sandflies. Whereas specimens retrieved from light traps produced intense and reproducible spectra that allowed reliable species determination, profiles of specimens from sticky traps were compromised and often did not enable correct identification. Sticky traps should therefore not be used in surveys that deploy MALDI-TOF MS protein profiling for species identification.

Key words. Phlebotomus, light traps, MALDI-TOF MS protein profiling, species identification, sticky traps, trapping methods.
transmission. Studies on sandfly biology, ecology and taxonomy are necessary to evaluate the risk for infection and to design control programmes in order to alleviate the burden caused by these diseases. As only some species have vectorial capacity to contribute to parasite transmission, the accurate identification of sandfly species is critical.

Unlike mosquitoes, in which surveillance efforts can target aquatic larvae in well-studied natural habitats and for which standardized larvae trapping methods have been developed, the larval stages of sandflies occur in a wide range of rather loosely defined habitats that are typically difficult to reach. Trapping efforts are therefore usually focused only on adults. Two main trap types are routinely applied in sandfly surveys: sticky traps made of oil-impregnated paper sheets, representing trapping by interception, and light traps that rely on attraction (Alexander, 2000). Both methodologies are well developed and offer advantages, as well as some drawbacks (Alten et al., 2015; Muñoz et al., 2017). The use of miniature Centers for Disease Control (CDC) light traps has gradually become a favourite method for sandfly trapping as these traps are less laborious to prepare and display, and are more versatile in different habitats than sticky traps, allowing better standardization of trapping. Moreover, as these light traps are commonly placed for short overnight periods, many specimens are collected alive, which is favourable for various assays including dissections to record Leishmania infections. Nevertheless, sticky traps remain a staple method of sandfly trapping in many field studies as they are cheap to prepare, do not rely on an external energy source and hence can be left for several days before collection and, importantly, are better suited to collect species with low phototropism (Alexander, 2000). Sandflies recovered from sticky traps are dead and impregnated in oil and must be transferred to storage vials using a fine brush dipped in ethanol in order to avoid damaging them.

The traditional method of species identification utilizes morphological characters on the head (cibarium and pharyngeal armature, antennal and palpal formula) and genitalia (arrangement of aedeagi and appendages in males, morphology of spermathcae in females) (Killick-Kendrick, 1990). This approach can be challenging and may eventually fail to lead to conclusive species identification because the assessment of these minute characters demands expertise and is time-consuming. Moreover, some field-collected specimens may be damaged and their identification may not be possible. Advances and the wide availability of molecular techniques in the last decades have offered alternative approaches to species identification. In addition to numerous DNA-based methods, matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) protein profiling has emerged as a molecular tool that can be successfully applied in the identification of various medically important arthropod vectors (Yssouf et al., 2016). This protein-based method identifies specimens by generating species-specific protein fingerprints and has become a popular, cost-effective, rapid and easy to perform method for accurate sandfly speciation (Dvorak et al., 2014; Halada et al., 2018).

Several aspects of MALDI-TOF MS protein profiling must be carefully addressed to obtain reproducible and species-specific spectra for robust and conclusive species identification. The body part, storage time and medium used, protein extraction method and the MALDI matrix type can affect the quality of the protein spectrum obtained (Yssouf et al., 2016). The capture method may also be important, but this has not been tested yet. The aim of this study was therefore to evaluate for the first time the suitability of MALDI-TOF protein profiling to identify sandfly species collected with the two most widely used trapping methods deployed in sandfly field surveys, CDC miniature light traps and sticky paper traps.

Sandflies included in the study originated from seven localities in Murcia Region in southeast Spain: Las Torres de Cotillas (38°01′42.3″ N, 01°14′40.6″ W); Molina de Segura (37°58′34.4″ N, 01°04′19.2″ W); Cobatillas (38°03′10.4″ N, 01°03′35.1″ W); Beniajan (37°58′34.4″ N, 01°04′19.2″ W); Archivel (38°04′22.1″ N, 02°00′23.3″ W); Yecua (38°36′44.4″ N, 01°06′41.7″ W), and Ceuti (38°04′39.6″ N, 01°16′27.0″ W). They had been collected in previous studies with miniature CDC light traps (John W. Hook Co., Gainesville, FL, U.S.A.) and castor oil-coated sticky traps placed for 24-h periods during late June and July in 2015 and 2016. Specimens yielded from both types of trap were stored in 70% ethanol at −20°C for 4 months or 17 months prior to morphological identification. Analysis by MALDI-TOF was carried out during October and November 2016.

For analysis, sandfly specimens were dissected with sterile micro-needles. Heads and terminal parts of abdomens bearing distinctive morphological characters (pharyngeal armature, cibarium, genitalia) were mounted in CMCP-10 medium (Polysciences Europe GmbH, Hirschberg, Germany) and identified using keys for sandflies of the Mediterranean region (Lewis, 1982; Killick-Kendrick et al., 1991).

Sample preparation and MALDI-TOF MS analysis followed a previously described protocol optimized for sandflies (Dvorak et al., 2014). Briefly, dissected thoraces were ground using a manual BioVortexer homogenizer (BioSpec Products, Inc., Bartlesville, OK, U.S.A.) with sterile disposable pestles in 10 μL of 25% formic acid that served as a homogenization solution. After short centrifugation (5000g for 15 s), 2 μL of the homogenate were mixed in a microtube with 2 μL of MALDI matrix and spotted directly on a steel MALDI plate. The MALDI matrix was prepared fresh as an aqueous 60% acetonitrile/0.3% TFA solution of sinapinic acid [30 mg/mL (Sigma-Aldrich Corp., St Louis, MO, U.S.A.)]. Protein mass spectra were measured on an Ultraflex III MALDI-TOF spectrometer (Bruker Daltonics, Inc., Billerica, MA, U.S.A.) and compared using FlexAnalysis 3.4 software (Bruker Daltonics, Inc.). For species identification, protein profiles were processed using MALDI Biotype 3.1 (Bruker Daltonics, Inc.) and queried against the present authors’ in-house database, which currently comprises reference spectra of 23 different sandfly species.

In total, 59 specimens (49 males, 10 females) were acquired; 29 were collected with CDC light traps and 30 with sticky traps placed at seven different localities in Spain. All specimens were conclusively identified by species-specific morphological features as belonging to the following five species: *Phlebotomus ariasi* (Tonnoir, 1921); *Phlebotomus papatasi* (Scopoli, 1786); *Phlebotomus perniciosus* (Newstead, 1911); *Phlebotomus sergenti* (Parrot, 1917), and * Sergentomyia minut a* (Rondani, 1843).
**Fig. 1.** Effect of trapping method on quality of MALDI-TOF mass spectra. Comparison of protein profiles of specimens of (A) *Phlebotomus perniciosus* and (B) *Sergentomyia minuta*, collected by Centers for Disease Control (CDC) light traps and sticky traps. By contrast with those collected in sticky traps, specimens caught in light traps produced intense spectra of better quality, allowing for conclusive species identification with higher log score values (LSVs).

Analysis by MALDI TOF of sandflies collected using CDC light traps gave intense and species-specific spectra of high quality (Fig. 1), resulting in correct species determination of 90% (*n* = 26/29) of specimens, including 100% (*n* = 14/14) with an average log score value (LSV) of 2.40 and 80% (*n* = 12/15) with an average LSV of 2.15 of those collected in 2016 and 2015, respectively, and an overall average LSV of 2.40 (an LSV of > 2.0 is accepted as indicating unambiguous assignment) (Table 1). Moreover, the analyses suggested the correct species for another three 2015 specimens, although the LSVs of these identifications were around 1.6 and hence below the threshold for conclusive identification. Therefore, these three specimens were classified as misidentified (Table 1). Other than this, the protein profiles of samples collected by light traps in 2015 were comparable with those of fresh individuals. Nevertheless, in several spectra, lower spectral intensity and loss of some peaks were observed, probably because of prolonged storage time.

By contrast, specimens collected by sticky traps produced spectra of visibly lower quality with elevated baselines and some missing signals, especially in the higher mass range (Fig. 1). Only 30% (*n* = 9/30) of these specimens were conclusively identified, including 60% (*n* = 9/15) with an average LSV of 2.10 from 2016 and 0% (*n* = 0/15) from 2015 (Table 1). The spectra of four 2016 specimens gave correct but unreliable assignments with LSV values of < 1.6, and two samples were misidentified within the same subgenus *Larroussius* (Table 1). Specimens from 2015 exhibited variable and markedly impaired spectra. Among them, two specimens provided correct yet uncertain species determination with LSV values of around 1.2. Further, the misidentifications included incorrect identifications of species that do not occur in the western Mediterranean, such as *Phlebotomus balcanicus*, *Phlebotomus perfiliewi* and *Phlebotomus similis*. Among the incorrect identifications, six specimens were assigned to an incorrect species within the same subgenus (*five* *Larroussius*, one *Paraphlebotomus*) and five individuals were assigned to a different subgenus (Table 1).

To fully understand sandfly–parasite interactions and transmission cycle dynamics, it is necessary to apply sampling strategies that provide representative numbers of insects in a condition that is adequate to enable successful species identification and further processing. Among the various trapping approaches, sticky traps have represented an indispensable and instrumental part of sandfly research in previous decades and have enabled the accumulation of vast amounts of data on sandfly fauna.

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Despite their obvious advantages, they gradually gave way to light traps, which rely on attraction rather than passive interception, and provide reliable estimates of the relative abundances of phototropic species, including *Leishmania* spp. vectors (Muñoz et al., 2017). Although they facilitate a partial catch of live specimens, one of the limitations of CDC light traps is their presumably selective attraction of different species and sexes (Alexander, 2000; Alten et al., 2015). Therefore, sticky traps have continued to be used in recent studies that aim to investigate sandfly fauna composition (Sawalha et al., 2017) and resting and breeding sites (Khan et al., 2017), or to detect the presence of *Leishmania* in captured females (Karakuş et al., 2017).

The technique of MALDI-TOF MS protein profiling represents an efficient approach towards species identification as this method has important benefits, including simple sample preparation, and extremely fast data acquisition and evaluation, and requires only inexpensive consumables. Although the acquisition of the necessary equipment can be prohibitively expensive, once it is available, the method is economically competitive with other molecular approaches, especially in entomological surveys in which financial and time constraints prevent the large-scale sequencing analysis of hundreds of field-collected specimens. As the MS-based technique does not require expertise in sandfly morphology, it may also rival traditional morphological analysis because it is more time-efficient and requires less expertise (Dvorak et al., 2014). Moreover, the data acquired by spectrometers produced by different manufacturers can be analysed using other software platforms (Mathis et al., 2015), which makes the method even more universal.

This is the first study to use MALDI-TOF MS protein profiling in field specimens caught by two different trapping methods in order to evaluate the effects of trap type on the quality of protein spectra and thus their potential for successful species identification. Specimens included in this study were collected in different habitats at seven periurban or agricultural sites in southeast Spain, where sandfly species composition, spatial distribution and ecological traits have been intensively studied (Muñoz et al., 2017, Risueño et al., 2017). Specimens of different sandfly species acquired by light traps produced protein profiles of quality sufficient to provide a high rate of successful identification and only three could not be unequivocally identified (Table 1).

By contrast, the rate of successful identification of specimens caught by sticky traps was much lower. The generally poor-quality spectra and failure to successfully identify specimens collected in sticky traps in 2015 may be partially attributed to prolonged storage time, which may have induced protein precipitation and decreased solubility, thus impairing spectral quality (Dvorak et al., 2014). However, time appeared to have less

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**Table 1.** Summary of sandfly specimens (*Phlebotomus* spp.) analysed and misidentified specimens.

| Country | Year of collection | Trap type | Specimens, n | Correctly identified, n | Misidentified, n | ID rate, % | Average LSV |
|---------|-------------------|-----------|--------------|------------------------|-----------------|------------|-------------|
| Spain   | 2016              | CDC       | 14           | 14                     | 0               | 100%       | 2.40        |
| Spain   | 2016              | ST        | 15           | 9                      | 6               | 60%        | 2.15        |
| Spain   | 2015              | CDC       | 15           | 12                     | 3               | 80%        | 2.10        |
| Spain   | 2015              | ST        | 15           | 0                      | 15              | 0%         | NA          |

- **Specimen code**
- **Locality**
- **Sex**
- **Year of collection**
- **Trap type**
- **ID by morphology**
- **ID by MALDI-TOF MS**
- **LSV**

CDC, Centers for Disease Control light trap; F, female; LSV, log score value; M, male; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; ST, sticky trap.
effect on specimens collected by CDC light traps as most samples from 2015 were correctly identified and produced spectra of markedly better quality than those of specimens collected in sticky traps.

In conclusion, the good quality of protein spectra from specimens captured by CDC light traps generally enabled correct species identification, whereas the lower quality of protein spectra from specimens collected by sticky traps resulted in numerous misidentifications. To date, specimen-associated parameters (i.e. used body parts, fed state), sample preparation procedures (sample storage, protein extraction, sample spotting method and the MALDI matrix used), and measurement parameters are recognized as important to the reproducibility of protein spectra analyses (Murugaiyan & Roesler, 2017). A standardized protocol of specimen collection also plays a key role in maintaining the integrity of a studied protein field sample and the most suitable trapping method should always be carefully considered.

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