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Identification of tick species and disseminate pathogen using hemolymph by MALDI-TOF MS

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ABSTRACT

Background: Matrix Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) is increasingly emerging tool for identification of arthropods including tick vectors using whole or body part of specimens. The challenges of the present study were to assess MALDI-TOF MS profiling for the both identification of tick species and *Rickettsia* spp. in infected ticks using hemolymph as protein mixture.

Methods: Firstly, hemolymph protein mixture from legs of 5 tick species, *Rhipicephalus sanguineus*, *Rhipicephalus bursa*, *Dermacentor marginatus*, *Hyalomma marginatum rufipes* and *Amblyomma variegatum* infected by *Rickettsia africae* were submitted to MALDI-TOF MS to assess tick species identification ability. Secondly, hemolymph MS spectra from *Rh. sanguineus* infected or not by *Rickettsia c. conorii* were compared to detect protein profiles changes. Finally, leg hemolymph MS spectra from new specimens of the 5 tick species were tested blindly including ticks infected by *R. c. conorii*. Discriminating mass peaks distinguishing the *R. c. conorii* infected and non-infected *Rh sanguineus* were determined.

Results: Consistent and reproducible MS profiles were obtained into each tick species. Comparison of MS spectra revealed distinct hemolymph protein profiles according to tick species. MS spectra changes were observed between hemolymphs from *R. c. conorii*-infected and non-infected *Rh. sanguineus* specimens, revealing 17 discriminating mass peaks. Clustering analysis based on MS protein profiles highlighted that hemolymph samples were grouped according to tick species. All tick hemolymph samples blindly tested against our home-made arthropod MS reference database were correctly identified at the species distinguishing also *R. c. conorii*-infected from *Rickettsia*-free *Rh. sanguineus* specimens.

Conclusion: The present study demonstrated the use of hemolymph MS profiles for dual identification of tick species and associated pathogens. This concomitant identification could be helpful for tick entomological diagnosis, notably for specimens removed directly on patients.

Introduction

Ticks are obligate hematophagous arthropods which have parasitized every class of vertebrates in almost world areas (Parola and Raoult, 2001). These ectoparasites may occasionally feed on humans and transmit a variety of pathogenic agents including viruses, protozoa and bacteria like *Rickettsia* spp. (Heyman et al., 2010; Hubalek and Rudolf, 2012; Parola et al., 2013). Several tick species are known as vectors of *Rickettsia* spp. (Parola et al., 2013). *Rhipicephalus sanguineus* appear as the main tick vector and potential reservoir of *Rickettsia conorii conorii*, the causative agent of the Mediterranean spotted fever (MSF) (Parola et al., 2013). MSF is endemic in the Mediterranean area, including northern Africa and southern Europe. Thus, the identification of ticks at the species level and the detection of *Rickettsia*-associated pathogen is indispensable for vectors monitoring and control of tick-borne rickettsiose in these regions (Parola et al., 2013).

Today, the reference methods for the tick species identification are generally determined either by using morphological criteria or by molecular methods (Tijsse-Klasen et al., 2014), and the routine method for the *Rickettsia* species identification in ticks remains the molecular biology (Parola and Raoult, 2001). The morphological identification of ticks is performed with taxonomic keys for endemic species according to geographical areas (Yssouf et al.,

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2013a). This taxonomic classification presents several limitations such as the availability of identification keys, an entomological expertise, and is powerless to distinguish sibling species (Karger et al., 2012). Moreover, this method could be insufficient for identification of damaged or engorged specimens by the loss of morphological criteria (Parola and Raoult, 2001). In addition, at immature tick stages, the lack of morphological criteria could prevent the accurate identification. Therefore, molecular approach remains the better strategy for identification of both tick species and associated *Rickettsia* spp. (Parola and Raoult, 2001). Nevertheless, DNA-based methods entail maximum time-consuming, are cost expensive for the consumables and requires information on gene sequences.

This last decade, a proteomic approach, the Matrix Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) technology was tested for identification and classification of some arthropod groups like *Drosophilia* (Feltens et al., 2010). More recently, MALDI-TOF MS was successfully employed for identification of hematophagous arthropods like *Culicoides* biting midges (Kaufmann et al., 2012), mosquitoes (Müller et al., 2013; Yssouf et al., 2013b, 2014a), tsetse flies (Hoppenheit et al., 2013), sand flies (Dvorak et al., 2014) and fleas (Yssouf et al., 2014b). This innovative proteomic strategy was presented as an alternative tool to discriminate tick species using whole specimens or body parts (Karger et al., 2012) such as legs only (Yssouf et al., 2013a). A recent pioneering study underlined that the submission of tick leg protein extracts to MALDI-TOF MS may allow one-step identification of tick species and tick-borne bacterial pathogens, including recurrent-fever *Borrelia* infecting *Ornithodoros* ticks (Fotso Fotso et al., 2014). More recently, MALDI-TOF MS was successfully applied for distinction of the presence of *R. c. conorii* or *R. slovaca* pathogens in *Rhipicephalus sanguineus* and *Dermacentor marginatus* using tick legs (Yssouf et al., 2015).

Ticks possess a circulatory system containing a circulating fluid, termed hemolymph. The hemolymph assay has long been used in live ticks to detect bacteria, particularly the spotted fever group Rickettsia (Burgdorfer, 1970). The distal portion of a leg is amputated, and the hemolymph that appears at the site can be smeared onto a microscope slide, stained by Gimenez and Giemsa staining or immuno-detection methods, and examined for the presence of bacteria (Parola and Raoult, 2001).

Thus, the aim of the present study was to investigate the use of MALDI-TOF MS to simultaneously identify the tick species and the presence of associated pathogens by using protein mixture of hemolymphs. The hemolymph MS protein profiles from 5 tick species were compared to determine the reproducibility and uniqueness of their respective MS protein profiles, taking into account their *Rickettsia* infectious status. Subsequently, the hemolymph MS spectra from the 5 tick species were added to our home-made arthropod reference MS spectra database, which was then used to identify new tick specimens by blind tests.

Materials and methods

Tick samples used for hemolymph extraction

Five adult tick species including *Rhipicephalus sanguineus*, *Rhipicephalus bursa*, *Dermacentor marginatus*, *Hyalomma marginatum rufipes* and *Amblyomma variegatum* infected by *Rickettsia africae*, laboratory-reared at the URMITE (Socolovschi et al., 2009), were used in the present study. All these tick species were collected in the field and laboratory breed. For detail about origins and collection time, see Table 1. The *D. marginatus* specimens were breaded in environmental incubator at 19 °C with a relative humidity of 80–90%, while the *Hy. m. rufipes*, *Rh. sanguineus* and *Rh. bursa* were maintained at 25 °C with a relative humidity of 80–90%. The *A. variegatum* specimens infected by *R. africae* were kept at 27 °C with a relative humidity of 60–70%. About *Rh. sanguineus* tick species, two "colonies" were available, one *Rickettsia*-pathogen free considered as non-infected and one *Rh. sanguineus* colony infected by *R. c. conorii*. Ticks infected by *Rickettsia* were reared in the biosafety level 3 (BSL3) laboratory in the same conditions as non-infected counterpart. The vertical transmission of *R. c. conorii* in *Rh. sanguineus* specimens and *R. africae* in *A. variegatum* specimens was controlled by molecular biology (Socolovschi et al., 2009).

Molecular detection of R. c. conorii *and* R. africae *in ticks*

DNA extraction was performed with one or two legs of each *Rh. sanguineus* and *A. variegatum* specimens included in the present study using the EZ1 DNA Tissue kit (Qiagen, Hilden, Germany). *R. c. conorii* and *R. africae* were detected by quantitative PCR using a CFX 96 Real Time System (BIO-RAD, Singapore) and Eurogentec MasterMix Probe PCR kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Specific primers and probes targeting a partial sequence of the tRNA intergenic spacer (IGS) and the citrate synthase A (*glt*A) encoding gene were used to detect, respectively, *R. c. conorii* and *R. africae* bacteria as previously described (Yssouf et al., 2014c).

R. conorii *culture and purification*

R. c. conorii (ATCC N° VR613) was grown into the cell line L929 (ATCC N° CCL-1) as previously described (Masala et al., 2012). Purification of the strain was performed according to the protocol described previously (Yssouf et al., 2014c). To eliminate

Table 1
Tick species used to establish the reference spectra database of leg hemolymphs and ticks used in blind test against leg hemolymph and previous homemade spectra database.

Species	Geographical origin (year of collection)	Number of specimens used for blind test	High LSVs obtained from blind tested against Database 1[a]	Number of specimens used for Database 1 upgrade	High LSVs obtained from blind tests against Database 2[b]
Rh. sanguineus	Southern France (2004)	2 (1F; 1M)	[1.522–1.668]	5 (4F; 1M)	[2.311–2.385]
Rh. sanguineus infected by *R. c. conorii*	Northern Algeria (2006)	3 (2F; 1M)	[1.143–1.457]	4 (3F; 1M)	[2.429–2.549][c]
Rh. bursa	Southern France (2013)	2 (2F)	[1.649–1.726]	5 (4F; 1M)	[2.536–2.617]
D. marginatus	Southern France (2014)	2 (1F; 1M)	[1.159–1.244]	5 (3F; 2M)	[2.007–2.379]
Hy. m. rufipes	Senegal (2014)	2 (2F)	[1.318–1.420]	4 (3F; 1M)	[1.810–2.437]
A variegatum infected by *R. africae*	Senegal (2013)	2 (2F)	[1.490–1.721]	5 (3F; 2M)	[2.404–2.498]
Total		13		28	

[a] The Database 1 is composed of MS spectra from 6 tick, 30 mosquito and 5 flea, 1 lice, 1 triatomines and 1 bedbug species.
[b] The Database 2 is composed of Database 1 plus hemolymph MS spectra from 5 tick species including specimens of *Rh. sanguineus* infected by *R. c. conorii*; for database details see materials and methods section.
[c] Matching with hemolymph MS spectra from *Rh. sanguineus* specimens infected by *R. c. conorii*. LSVs, log score values; M, male; F, female.