A proteomic approach to the identification of salivary proteins from the argasid ticks *Ornithodoros moubata* and *Ornithodoros erraticus*

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Abstract

The saliva of ticks contains anti-haemostatic, anti-inflammatory and immunomodulatory molecules that allow these parasites to obtain a blood meal from the host and help tick-borne pathogens to infect the vertebrate host more efficiently. This makes the salivary molecules attractive targets to control ticks and tick-borne pathogens. Although *Ornithodoros moubata* and *O. erraticus* are important argasid ticks that transmit severe diseases, to date only a few of their salivary proteins have been identified. Here we report our initial studies using proteomic approaches to characterize the protein profiles of salivary gland extracts (SGE) from these two argasids. The present work describes the proteome of the SGEs of both tick species, their antigenic spots, and the identification of several of their proteins. The whole number of identifications was low despite the good general quality of the peptide mass maps obtained. In the *O. moubata* SGE, 18 isoforms of a protein similar to *O. savignyi* TSGP1 were identified. In the *O. erraticus* SGE we identified 6 novel proteins similar to unknown secreted protein DS-1 precursor, NADPH dehydrogenase subunit 5, proteasome alpha subunit, ATP synthase F0 subunit 6, lipocalin and alpha tubulin. Finally, the current drawbacks of proteomics when applied to the identification of acarine peptides and proteins are discussed.

Keywords: *Ornithodoros*, salivary gland extract, saliva, proteome, mass spectrometry

1. Introduction

Ticks are ectoparasitic, haematophagous arthropods whose public health importance resides in their ability to transmit a large variety of infectious agents to humans and domestic animals (Jongejan and Uilenberg, 2004). The saliva of the ticks is known to contain anti-haemostatic, anti-inflammatory and immunomodulatory molecules that modify the physiology of their vertebrate hosts at the tick bite site, allowing these parasites to obtain a blood meal from the host (Valenzuela, 2002, 2004). Tick-borne pathogens can take advantage of this modification to infect the vertebrate host more efficiently (Nuttal and Labuda, 2004). With non-natural hosts, however, tick feeding often induces immune and allergic responses, presumably to the salivary proteins, resulting in tick rejection (see review by Brossard and Wikel, 2004) and, interestingly, in some cases immune responses to tick saliva confer protection to pathogen transmission (Nuttall and Labuda, 2004). Accordingly, knowledge of the salivary components in vector ticks can lead to the discovery of novel pharmacological molecules and to the development of novel anti-tick vaccines and even vaccines based on tick salivary proteins to control tick-borne diseases (Willadsen, 2004; Nuttall et al. 2006; Ribeiro et al., 2006; Titus et al., 2006).

Soft ticks (Argasidae) and hard ticks (Ixodidae) are different as regards their feeding habits and the complexity of their salivary glands. Argasids feed for a short period only (a few minutes to an hour), take less blood than 12 times their unfed weight, and do not secrete cement. Ixodids feed for several days, secrete cement to assist attachment, and ingest enormous amounts of blood (up to 100 times their unfed weight). In addition, they use their salivary glands in osmoregulation by returning about 70% of the water and ion content of the blood-meal back to the host by salivation into the feeding site. Thus, the salivary gland structure and salivary composition of ixodid ticks are more complex than those of argasid ticks (see Bowman and Sauer, 2004).

In ixodid ticks, a number of salivary pharmacologically active proteins have been already identified, as shown by the latest reviews on this topic (Basanova et al., 2002; Mans and Neitz, 2004; Valenzuela 2004; Steen et al., 2006). In argasids, however, the number of known salivary proteins is considerably smaller, and most of them have been identified essentially from two species, the African ticks *Ornithodoros savignyi* and *O. moubata*.

Although the ticks studied here were *O. moubata* and *O. erraticus*, we believe it appropriate to mention the known salivary proteins of *O. savignyi* too, because of the importance of this species and its phylogenetic relatedness to *O. moubata* (Mans et al., 2003). *O. savignyi* produces paralysis and toxicosis, which makes it a serious danger for domestic animals in South African countries (Mans et al., 2002). The salivary secretion of *O. savignyi* is known to contain the so-called Tick Salivary Gland Proteins (TSGPs), which belong to the lipocalin family, and are the most highly abundant proteins in the salivary gland extract (SGE) of this tick and have been proposed to play a role in salivary gland granule biogenesis. There are no known anti-haemostatic activities associated with TSGPs, but TSGP2 and TSGP4 have been identified as toxins that affect the cardiovascular system of the host (Mans et al., 2001, 2002, 2003, 2004). Apart from TSGPs, several anti-haemostatic components have been identified in the salivary secretion of *O. savignyi* (see Valenzuela, 2004). These are two anti-platelet factors (apyrase and Savignyigrin) and four anticoagulants (Savignin -anti-thrombin-, the inhibitor of the fXa and the so-called BSAP1 and BASP2, which inhibit the extrinsic pathway).

Regarding *O. moubata*, this is a very important species because it transmits the agent of the African human relapsing fever -*Borrelia duttoni*- and the African swine fever virus, which, together with the large size and ease of management of this tick, make it a good model for the study of vector-pathogen relationships (Nakajima et al., 2003; Saravanan et al., 2003). Nevertheless, only a few of their salivary proteins have been identified. These include four anti-platelet factors (apyrase, Disagregin, Moubatin and the Tick adhesion inhibitor), two anti-clotting factors (the Tick anticoagulant peptide -anti-fXa- and Ornithodorin -anti-thrombin-), and an inhibitor of the complement component C5 (OmCI), discovered more recently (Nunn et al., 2005). To these proteins must be added one more: an antigenic protein (20A1) identified in

the SGE by Baranda et al. (2000), whose N-terminal amino acid sequence shows 50% identity with the TSGP1 of *O. savignyi* (Mans et al., 2001).

Besides *O. moubata*, we were also interested in *O. erraticus* because it is present in the Iberian Peninsula and also transmits African swine fever virus (Wilkinson, 1984; Basto et al., 2006), together with several species of tick-borne relapsing fever borreliae, such as *B. hispanica* and *B. crocidurae* (Piesman and Gage, 2004). Accordingly, the saliva and SGE of *O. erraticus* have been examined by our team to look for their anti-inflammatory and immunomodulatory activities (Astigarraga et al., 1997), as well as to search for diagnostic (Baranda et al., 1997, 2000) and protective antigens (Astigarraga et al., 1995). These studies have revealed several immunomodulatory mechanisms (inhibition of complement activation, inhibition of the synthesis of specific anti-tick IgE) and have shown that it is possible to induce partly protective immune responses using salivary antigens. On the other hand, however, such studies have been poorly successful in the molecular identification of *O. erraticus* salivary molecules since only one antigenic protein has been purified and its N-terminus sequenced, showing 81-91% similarity to the haemoglobin alpha chain of several species of mammals (Baranda et al., 2000). The other salivary molecules remain to be identified.

It can thus be said that the salivary components of *O. moubata*, and especially those of *O. erraticus*, remain insufficiently investigated. Accordingly, in order to expand our knowledge about the salivary components of these two ticks, we applied a proteomic approach (two-dimensional gel electrophoresis, mass spectrometry and *de novo* sequencing) to the identification of the proteins present in the SGEs of both tick species. The work reported here describes the salivary proteome of both ticks, their antigenic spots, and the identification of several of their salivary proteins. Finally, the current drawbacks of proteomics when applied to the identification of acarine peptides and proteins are discussed.

2. Material and methods

2.1. Ticks

The *O. erraticus* and *O. moubata* ticks used in this work came from two colonies maintained in our laboratory. The colony of *O. erraticus* was established from specimens captured in Salamanca, western Spain, and the colony of *O. moubata* was established from specimens submitted from the Institute for Animal Health, Pirbright, Surrey, UK. The ticks in those colonies are fed regularly on rabbits and kept at 28 ºC, 85 % relative humidity and 16 h light / 8 h darkness.

2.2. Collection of salivary gland extracts (SGE) and preparation of samples for two-dimensional gel-electrophoresis

The SGEs were prepared in batches from 50 unfed adult ticks (males and females at an equal proportion) as described elsewhere (Oleaga-Pérez et al., 1994). Briefly, salivary glands were extracted in phosphate-buffered saline, pH 7.4 (PBS) at 4 ºC, rinsed three times with fresh PBS, and suspended in 2 ml of PBS. Then, they were frozen (-20 ºC) and thawed five times and centrifuged for 30 min at 18,000 g. The supernatant was recovered, filtered through a 0.2 µm filter, and termed SGE. The protein concentrations of the SGEs were measured with the Bradford assay (Bio-Rad, Hercules, CA, USA).

The SGE samples to be electrophoresed were previously desalted and concentrated with the ReadyPrep 2-D Cleanup Kit (Bio-Rad), following the manufacturer's instructions. In this way, samples of SGE containing 400 µg of protein were cleaned and resuspended in 1250 µl of 2-D rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 50 mM DTT) without ampholytes. The sample was divided into 125 µl aliquots (containing 40 µg of protein) and these were kept at -80 ºC until used.

2.3. Two-dimensional electrophoresis

Isoelectric focusing (IEF) was performed in 7 cm immobilised pH gradient (IPG) strips with linear pH ranges of 3-10, 5-8 and 7-10 (Bio-Rad) using a Protean IEF Cell (Bio-Rad) with a surface temperature of 20 ºC and a maximum current of 50 µA/strip.

The 125 µl sample aliquots were thawed and each of them was supplemented with 1.25 µl of 100x Bio-Lyte ampholytes (Bio-Rad) of the respective pH range to reach a final concentration of 0.2% ampholytes. Following this, the samples were allowed to mix gently for 1 h at room temperature before centrifugation at 18,000 *g* over 30 min to remove all particulate material. The supernatants were applied to the IPG strips by in-gel rehydration at 20ºC for at least 12 h, after which IEF was run for a total of 15,000 Vh.

Following IEF, the strips were reduced in equilibration buffer (6 M urea, 0.05 M Tris, pH 8.8, 2% SDS and 20% glycerol) containing 2% DTT over 15 min and then alkylated in equilibration buffer containing 2.5% iodoacetamide for 10 min. The second dimension was performed on 12% SDS-polyacrylamide gels using a Mini Protean cell (Bio-Rad). Running proceeded at 15 mA/gel for the first 15 min and then at 30 mA/gel. After running, the 2-D gels were either stained or electroblotted onto nitrocellulose membranes for immunoblot analysis.

2.4. Protein staining and image analysis

The 2-D gels were routinely stained with silver stain or with a mass-compatible silver stain when the gel was to be used for mass spectrometry analysis (Stochaj et al., 2003). Additional 2-D gels were stained with Sypro Ruby florescent dye (Sigma, Saint Louis, MO, USA) according to the manufacturer's instructions for quantitative analysis.

Sypro Ruby-stained gels were digitalized with the Fluor-S Multimager system (Bio-Rad) and silver-stained gels were scanned using an ImageScanner (Amersham Biosciences, Uppsala, Sweden). Analysis of 2-D gel images was accomplished using ImageMaster 2D Platinum Software v5.0 (Amersham Biosciences), including the assessment of the relative abundance of all the spots detected in the Sypro Ruby-stained 2-D gels.

2.5. Immunoblot analysis

Proteins were electrotransferred from 2-D gels to nitrocellulose membranes at 400 mA for 90 min. Blots were blocked with 1% BSA in PBS for 1 h and then rinsed with PBS containing 0.05% Tween 20. Following this, they were incubated with anti-*O. erraticus* or anti-*O. moubata* pig sera or with pig naïve sera at 1:50 dilution. These sera were obtained in earlier works, from pigs infested with each tick species, and preserved at -80 ºC. After three new washes, the blots were incubated with a horseradish peroxidase-labelled anti-pig IgG (Sigma) at 1:2,000 dilution and washed again three times. Incubations were performed at 37 ºC for 1 h, and the washes were carried out at room temperature for 10 min per each wash. Finally, the antigenic spots were developed with 4-Cl-1-naphthol and their images were scanned with the ImageScanner (Amersham Biosciences). Immunoblots and their homologous silver-stained gels were aligned to isoelectric point (pI) and molecular weight (MW) and then matched by ImageMaster software in order to identify the antigenic spots in the gels.

2.6. In-gel digestion of proteins for mass spectrometry analysis

Selected spots from each extract (the antigenic ones and the more abundant non-antigenic spots) were excised manually from the gels and subjected to mass spectrometry analysis. Protein spots were digested automatically using a Proteineer DP protein digestion station (Bruker-Daltonics, Bremen, Germany) following the protocol of Shevchenko et al. (1996) with minor variations: gel plugs were subjected to reduction with 10 mM DTT and alkylation with 55 mM iodoacetamide, both in 50 mM ammonium bicarbonate. The gel pieces were then rinsed with 50 mM ammonium bicarbonate and acetonitrile and dried under a stream of nitrogen. Modified porcine trypsin at a final concentration of 13 ng/µl in 50 mM ammonium bicarbonate was added to the dry pieces of gel and the digestion proceeded at 37ºC for 6 h. Finally, 0.5% trifluoroacetic acid was added for peptide extraction.

2.7. MALDI-MS(/MS)

An aliquot of the above digestion solution was mixed with an aliquot of α-cyano-4-hydroxycinnamic acid in 33% aqueous acetonitrile and 0.1% trifluoroacetic acid. This mixture was deposited onto a 600 µm AnchorChip MALDI probe (Bruker-Daltonics) and allowed to dry at room temperature. MALDI MS(/MS) data were obtained using an Ultraflex time-of-flight mass spectrometer (Bruker-Daltonics) equipped with a LIFT-MS/MS device (Suckau et al., 2003). Spectra were acquired in the positive-ion mode at 50 Hz laser frequency, and 100 to 1500 individual spectra were averaged. For fragment ion analysis in the tandem time-of-flight (TOF/TOF) mode, precursors were accelerated to 8 kV and selected in a timed ion gate. Fragment ions generated by laser-induced decomposition of the precursor were further accelerated by 19 kV in the LIFT cell and their masses were analyzed after passing the ion reflector. Measurements were in part performed using post-LIFT metastable suppression, which allowed removal of precursor and metastable ion signals produced after extraction out of the second ion source. Detailed analysis of peptide mass mapping data was performed using flexAnalysis software (Bruker-Daltonics). Internal calibration of MALDI-TOF mass spectra was performed using two trypsin autolysis ions with m/z = 842.510 and m/z = 2211.105; for MALDI-MS/MS, calibrations were performed with fragment ion spectra obtained for the proton adducts of a peptide mixture covering the 800-3200 m/z region.

For selected samples, peptide sequences were deduced by *de novo* sequencing based on the MALDI-MS/MS data. Deduced peptide sequences were then compared to databases for identification (see below).

2.8. nLC-MS/MS and de novo sequencing.

Selected spots of *O. erraticus* whose identification remained elusive were re-analysed by nLC-MS/MS and *de novo* sequencing. These spots were excised from fresh gels and incubated for several minutes in ultra-pure water. Then, they were digested according to the

protocol referred to in section 2.6. The resulting tryptic peptides were on-line injected into a C-18 reversed-phase nano-column (Discovery® BIO Wide pore, Supelco, Bellafonte, PA) and analyzed in a continuous acetonitrile gradient. A flow rate of 300 nl/min was used to elute the peptides from the reversed-phase nano-column to an electrospray ion source coupled to an ion trap mass spectrometer (Esquire HCT, Bruker-Daltonics) for real-time ionization and fragmentation. For all spots, *de novo* tentative sequences were obtained using the comprehensive analysis of the corresponding MS/MS spectra.

2.9. Database searching

MALDI-MS and MS/MS data were combined through MS BioTools program (Bruker-Daltonics) to search the NCBInr database using Mascot software (Matrix Science, London, UK; Perkins et al., 1999).

Peptide sequences obtained by *de novo* sequencing were submitted to the BLAST search algorithm at the NCBI (http://www.ncbi.nlm.nih.gov/BLAST/). Additionally, all these peptide sequences were compared with virtual translations of the sequences deposited in the currently available tick EST databases at The Gen Index databases (http://compbio.dfci.harvard.edu/tgi/), namely, *Amblyomma variegatum*, *Boophilus microplus, Ixodes scapularis* and *Riphicephalus appendiculatus*. Each peptide sequence was submitted through Gene Indices, BLAST, and then compared simultaneously with the four tick databases using the tBLASTn program.

3. Results

3.1. Two-dimensional gel analysis of the O. moubata SGE

The SGE of *O. moubata* was first resolved in 2D gels of pH range 3-10 and 12% polyacrylamide. Samples were analysed in triplicate to assess overall reproducibility of the

protein spot patterns and only minor differences were apparent between gel replicates. Staining of these gels with silver nitrate permitted the detection of 70 spots between a pI range of 5-9 and a MW 13-90 kDa (Fig. 1A). Notably, the more intense spots localized close to each other in a small area of the 2D gel: between pI 5.0-5.8 and MW 22-31 kDa. Quantitative analysis of similar gels stained with Sypro Ruby showed that these intense spots were in fact the most abundant ones, representing more than 90% of the protein mass of the SGE (Fig. 1B). In order to improve the resolution of these major spots and to increase the number of spots detected, the SGE was then resolved in 2D gels in the 5–8 and 7–10 pH ranges, and these gels were stained with silver nitrate. In this way, however, only 5 additional spots were detected and the resolution between the major spots was scarcely improved (not shown). Therefore, the subsequent immunoblot analysis of the *O. moubata* SGE was carried out in 2D gels in the 3-10 pH range.

3.2. Antigenic spots of the O. moubata SGE

As shown in Fig. 1C, the 2D immunoblot analysis of the SGE with sera from pigs bitten by *O. moubata* revealed around 18 major antigenic spots in the range of pI 5.1-6.3 and 24-31 kDa, and some minor and poorly resolved spots in the range of pI 5.5-5.9 and 64-73 kDa. Sera from naïve pigs did not reveal any spots on the 2D immunoblot (not shown). Matching of the immunoblot with its homologous silver-stained gel allowed us to localize the 18 major antigenic spots in the 2-D gels (Fig. 1D and 1E) and revealed that these antigenic spots were indeed some of the most highly abundant spots of the SGE (spots No. 6 to 23).

3.3. Identification of O. moubata salivary proteins

A total of 40 spots were cut from silver-stained 2D gels (of pH range 3-10) and subjected to MS analysis: the 18 major antigenic spots (Fig. 1D) and 22 additional non-antigenic spots (Fig. 1A). Up to 20 of these 40 spots were identified as tick proteins (Table 1).

Peptide mass fingerprints (PMF) were obtained for all 40 spots, although none of them could be unambiguously identified on the basis of its PMF. Noticeably, the PMFs of the 18 major antigenic spots (No. 6 to 23) were identical to one another, suggesting that all these spots were isoforms of the same protein (Fig. 2). Likewise, the PMFs of spots No. 2 and 3 were identical to each other, and this was also the case for spots No. 4 and 5 and for spots No. 37 and 38, respectively (not shown).

Tandem mass spectrometry (MS/MS) followed by an MS/MS ion search of the NCBInr database resulted in the identification of only two spots; namely, the non-antigenic spots No. 4 and 5. As suspected from their identical PMFs, both spots contained two isoforms of the same protein: the Moubatin precursor (Table 1).

De novo sequencing based on MALDI-MS/MS data provided the amino acid sequence of two peptides derived from spot No. 13 (Fig. 2). BLAST searching of the NCBInr database with these sequences identified spot No. 13 as being similar to Tick Salivary Gland Protein 1 (TSGP1) from *O. savignyi* (Table 1). Since spots No. 6 to 23 had identical PMFs to that of spot No. 13, it could be assumed that all of them contained isoforms of TSGP1. As described in section 3.2., all these TSGP1 isoforms were antigenic.

No significant alignments were observed between the deduced peptide sequences of *O. moubata* and the sequences deposited in the tick EST databases.

3.4. Two-dimensional gel analysis of the O. erraticus SGE

As done with that of *O. moubata*, the SGE of *O. erraticus* was first resolved in 2D gels with a pH range of 3-10 and 12% polyacrylamide. Samples of *O. erraticus* SGE were also analysed in triplicate to assess reproducibility and only minor differences were apparent between gel replicates. Staining of these gels with silver nitrate permitted the detection of around 180 spots, most of which were located between pH 5 and 9 (only two spots with a pI < 5 were observed) over a broad range of MW (19-114 kDa) (Fig. 3A). Quantitative analysis of similar gels stained with Sypro Ruby showed that there were around 13 highly abundant spots,

representing 82% of the protein mass of the SGE. These spots localized along a broad range of pI (4.9-8.4), but within a narrow MW range (23.2-27.5 kDa) (Fig. 3B). Once we had determined the spot pI and MW ranges, in order to improve spot resolution and detection the SGE was resolved in 2D gels with pH ranges of 5-8 and 7-10. As may be seen in Fig. 3C and 3D, the silver stain detected a higher number of spots (223) and with a significantly improved resolution. Consequently, the subsequent immunoblot analysis of the *O. erraticus* SGE was carried out in 2D gels in the 5-8 and 7-10 pH ranges.

3.5. Antigenic spots of the O. erraticus SGE

As shown in Fig. 3E and 3F, 2D immunoblot analysis of the SGE with sera from pigs bitten by *O. erraticus* revealed approximately 59 antigenic spots, most of them in the pI range of 5-8 but clearly distributed in two ranges of MW, from 21 to 25 kDa and from 49 to 106 kDa. Sera from naïve pigs did not reveal any spots on the 2D immunoblot (not shown). Matching of the immunoblots with their homologous silver-stained gels allowed us to localize up to 27 of the 59 antigenic spots in the 2D gels (Fig. 3C and 3D). These spots were cut from the gels and subjected to mass spectrometric analysis (see below).

3.6. Identification of O. erraticus salivary proteins

A total of 56 spots were cut from silver stained 2D gels and subjected to MS analysis: 27 antigenic spots and 29 non antigenic spots. All these spots were cut from 2D gels of pH ranges 5-8 and 7-10 (Fig. 3C and 3D) except spot No. 41, which had a pI < 5 and had to be cut from a 2D gel of pH 3-10 (Fig. 3A).

Only 7 of these spots were identified as parasite proteins (Tables 2 and 3). Peptide mass fingerprints (PMF) were obtained for all 56 spots, although none of them could be unambiguously identified on the basis of its PMF. Tandem mass spectrometry (MS/MS) followed by an MS/MS ion search of databases did not result in the identification of any protein

either. Accordingly, we attempted the identification by *de novo* sequencing for some spots selected on the basis of their antigenicity or high abundance.

De novo sequencing based on MALDI-MS/MS data provided the amino acid sequences of 4 peptides (1 peptide per spot) of spots No. 22, 25, 41 and 49 (Table 2). BLAST searching in the NCBInr protein database using these sequences led to the identification of 4 different proteins: NADPH dehydrogenase subunit 5, ATP synthase F0 subunit 6, unknown secreted protein DS-1 precursor and proteasome alpha subunit. Similar searches in the tick EST databases revealed significant matching for only one peptide sequence -that of the proteasome alpha subunit- which displayed 100% similarity with the corresponding fragment in the proteasome alpha subunit of *Amblyomma variegatum*.

D*e novo* sequencing based on LC-MS/MS data provided the amino acid sequences of a total of 15 peptides from spots No. 45, 47, 48, 53 and 54 (Table 3). A single peptide from spot No. 45 and three peptides from spot No. 54 were sequenced, but their amino acid sequences did not align significantly with any sequence from the NCBInr database nor from the tick EST databases. Four peptides from each of spots No. 47 and 48 were sequenced and the deduced peptide sequences were exactly the same in both spots. Only one of these four peptide sequences aligned significantly (100%) with the lipocalin sequence from *Argas monolakensis*. Finally, the amino acid sequences of three peptides from spot No. 53 were obtained. Two of them aligned significantly (100% and 87.5%) with the alpha tubulin sequence from *Drosophila melanogaster*. In addition, one of these two peptides was identified in the *Ixodes scapularis* and *A. variegatum* EST databases as alpha tubulin, with 90% sequence similarity.

4. Discussion

Due to the importance of tick saliva in blood feeding, host immunity, and pathogen transmission, our aim in this work was to identify and characterize the salivary proteins of *O. moubata* and *O. erraticus*. To accomplish this goal we carried out a proteomic study of the salivary gland extracts (SGE) of both ticks based on 2D-electrophoresis, mass spectrometry and

database searching, in a similar way to that of the scant proteomic studies carried out with tick saliva and salivary gland extracts (Mans et al., 2001; Madden et al., 2002) or with extracts of whole tick larvae (Untalan et al., 2005).

As a source of salivary proteins, we obtained SGEs instead of saliva owing to the low protein yield of the laborious, time-consuming protocol for saliva collection as compared to that of SGE preparation (2.1 *vs*. 150 µg/specimen in *O. moubata* and 0.4 *vs*. 50 µg/specimen in *O. erraticus*, Baranda et al., 1997). We then constructed the proteomic maps of the SGEs of *O. moubata* and of *O. erraticus*, including the assessment of the most abundant and the antigenic spots, and finally, we achieved the identification of 20 spots from *O. moubata* and 7 spots from *O. erraticus*.

The proteomic maps of the *O. moubata* and *O. erraticus* SGEs showed significant differences between them. Regarding the total number of spots detected, the proteome of the *O. moubata* SGE showed a significantly lower number (70 spots in Fig. 1A and 5 additional spots detected in 2D-gels of pH 5-8 and 7-10, not shown) than that of the *O. erraticus* SGE (223 spots, Fig. 3). This suggests that the SGE of *O. moubata* has a simpler composition than that of *O. erraticus*. Although this is the first proteomic study carried out with the *O. erraticus* SGE, the same is not the case for *O. moubata*, since Mans et al. (2004) have already provided 2D-images of its SGE proteome and also of the SGE proteome of *O. savignyi*. Those authors compared both proteomes and observed that the highly abundant proteins in the SGE from *O. moubata* had similar MWs and pIs to the TSGP1-3 from *O. savignyi*, although they did not provide further information about the other spots in the proteome of the *O. moubata* SGE. The 2D-image of the *O. moubata* SGE proteome reported by Mans et al. (2004) is similar to that reported by us, although some differences between them can be observed in the number, resolution and distribution of the spots. These discrepancies are possibly due to differences in the amount of SGE loaded, the staining protocol, and the type of IPG strip used (non-linear pH 3-10 *vs*. linear pH 3-10).

The SGEs of *O. moubata* and *O. erraticus* differed in the number and distribution of the antigenic spots detected by the sera of the infested pigs. Notwithstanding, in both ticks the ratio

of antigenic spots to the total number of spots was low (approximately 24% for *O. moubata* and 26% for *O. erraticus*). This would mean that most of the salivary components of these two *Ornithodoros* are not immunogenic. According to Astigarraga et al. (1997), this lack of immunogenicity would be a strategy used by the tick to evade the host immune system. Those authors suggested that the non-antigenic salivary proteins would be those that play relevant functions during blood feeding, such that their blockade with antibodies could affect the parasite negatively and hence these would have eliminated the immunogenicity of these salivary proteins during tick-host coevolution. An example of such a non-antigenic pharmacologically active salivary protein is the Moubatin of *O. moubata* (see below).

Regarding the spots identified, the analysis by MS allowed us to identify, in the set of both species, 27 of the 96 spots analyzed, which in turn corresponded to 8 proteins: 2 from the SGE of *O. moubata* and 6 from the SGE of *O. erraticus*.

Regarding *O. moubata*, we analyzed 40 spots (18 antigenic and 22 non-antigenic) by MS and identified up to 20 spots that corresponded to only two proteins (Table 1). The only two non-antigenic spots identified (spots No. 4 and 5) were isoforms of the Moubatin precursor. Moubatin is a 17 kDa protein of *O. moubata,* already known and sequenced (Keller et al., 1993), that belongs to the lipocalin family. It is an anti-platelet factor that inhibits the platelet aggregation induced by collagen; although its mechanism of action is not clear, it has been suggested that Moubatin may bind ADP with high affinity, preventing the platelet aggregation caused by ADP released from collagen-activated platelets (see Valenzuela, 2004). On the other hand, the most striking feature of the immunome of the SGE of *O. moubata* was that all the antigenic spots (18 spots) were isoforms of a single protein. This protein, similar to the TSGP1 of *O. savignyi*, was unequivocally identified after comparing peptide sequences deduced by *de novo* sequencing. The protein is clearly the most abundant one in SGE of *O. moubata* and all its isoforms show acid pIs, like the TSGP1-3 of *O. savignyi*. Accordingly, it could be suggested that the TSGP1 of *O. moubata* could be involved in tick salivary gland granule biogenesis as are the TSGPs of *O. savignyi* (Mans et al., 2001). Likewise, it is reasonable to speculate that the antigen 20A1, previously identified by Baranda et al. (2000) in the SGE of *O. moubata*, would

be one of the many isoforms of TSGP1 identified here. The presence of all these isoforms indicates that there is a large degree of redundancy among the salivary proteins of this tick species. This phenomenon is not exclusive to *O. moubata* since a similar one, although at the level of cDNA, was observed upon studying the sialome of *Argas monolakensis* (Mans et al., 2006) and the transcriptomes of the salivary glands of *Ixodes pacificus* (Francischetti et al., 2005), *I. scapularis* (Ribeiro et al., 2006) and *Dermacentor andersoni* (Alarcon-Chaidez et al., 2007). Some authors have suggested that redundancy of isoforms of pharmacologically active salivary proteins could help the tick to feed on different species of vertebrate hosts or to evade the immune response by means of mechanisms of antigenic variation (Francischetti et al., 2005; Ribeiro et al., 2006). In the particular case of the TSGP1 of *O. moubata*, we still do not know what the biological significance of the existence of many isoforms might mean.

Finally, with respect to this species it was somewhat surprising not to find its reported pharmacologically active salivary proteins in the SGE, except Moubatin, even though we analysed almost all the spots developed. A possible explanation for this is that such proteins maybe masked by abundant, immunodominant proteins (such as TSGP1) or simply not detectable by the methods used.

Regarding *O. erraticus*, we analyzed by MS 56 spots (27 antigenic and 29 non-antigenic) but we identified only 7 spots that corresponded to 6 proteins (Tables 2 and 3). None of these proteins seemed to have functions related to anti-haemostatic, anti-inflammatory or immunomodulatory activities. The unknown secreted protein DS-1 precursor is a hypothetical protein translated from the nucleotide sequence of gut-specific genes in *O. moubata* whose function remains to be elucidated (Sojka et al., 2004). Since we identified it in a salivary extract, this suggests that as well as in the gut it would also be expressed in the salivary glands, as is the case of other *O. moubata* proteins, such as the novel galectin OmGalec (Huang et al., 2007). In turn, the proteasome alpha subunit, the NADPH dehydrogenase subunit 5 and the ATP synthase F0 subunit 6 have housekeeping functions, such that their presence in the saliva seems debatable. Nevertheless, the latter two are antigenic, indicating that they really are inoculated into the host with the saliva.

Another two antigenic proteins identified were lipocalin and alpha tubulin. The two antigenic isoforms of lipocalin identified (Table 3) were highly abundant in the SGE and showed acidic pIs and MWs in the same range as those of the TSGP1-3 of *O. savignyi* and the TSGP1 of *O. moubata*. This suggests that they could also be involved in salivary gland granule biogenesis. Evidently, demonstration of this aspect requires more studies.

Tubulin was identified in one spot on the basis of the sequence identity of two of the three peptides subjected to *de novo* sequencing. This *O. erraticus* tubulin had a very different MW to that of *D. melanogaster* (Table 3) raising some doubts as to its real identity. However, the identification was reinforced by the 90% similarity of one of these two peptides with the sequences of alpha tubulin from *Amblyomma variegatum* and *I. scapularis* in their EST databases. Since alpha tubulin is a structural protein, its presence (and potential roles) in tick saliva is rather surprising. For the time being, all we can say is that this protein really must be inoculated to the host with the tick saliva because it is recognized by the sera of pigs bitten by *O. erraticus*.

4. Conclusions

Here we report our initial studies using proteomic approaches to characterize the protein profiles of salivary gland extracts from two argasid tick species, *O. moubata* and *O. erraticus*. We present the 2D electrophoretic protein expression maps of these SGEs, obtaining peptide mass maps and peptide sequence information that have allowed us to identify some of the more abundant and antigenic proteins of these argasids. The number of unambiguous identifications (27 spots identified *vs*. 96 analyzed, 28%) was rather low, despite the good general quality of the MS and MS/MS spectra obtained. In fact, most of the identifications were only achieved after similarity searches of peptide sequences obtained by *de novo* sequencing. The limited number of acarine sequences currently available in public databases was a serious drawback to the identification of tick salivary proteins using proteomic approaches based on MALDI-MS(/MS) data and was also a limitation when conducting sequence similarity searches.

However, the number of such sequences is rapidly growing thanks to the recent application of comprehensive high-throughput approaches to the analysis of tick salivary transcriptomes.

It is our intention to continue the analysis of the sialomes of these two *Ornithodoros* species, but at the cDNA level instead of that of expressed proteins, in order to gain a resource that could be used in future studies on the biological role of such salivary proteins and for the development of novel control strategies of these ticks and the pathogens they transmit.

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Figure captions

Fig. 1. Representative two-dimensional images of the SGE from *O. moubata*. All the gels in this figure were in the 3-10 pH range and 12% polyacrylamide. Reference molecular masses are indicated on the left. (A) Silver-stained gel. The non-antigenic spots that were analyzed by mass spectrometry are circled and numbered in black. (B) Sypro Ruby-stained gel showing the most abundant proteins in the SGE. (C) 2D-Western blot showing the antigenic spots revealed by a pool of sera from pigs experimentally infested with *O. moubata*. (D and E) Magnifications of panels A and C showing the antigenic spots analyzed by mass spectrometry (white numbered circles).

Fig. 2. Representative PMF spectrum of spots No. 6 to 23. *De novo* sequencing based on MALDI-MS/MS data allowed elucidation of the amino acid sequences of peptides A (YPYYMGYK) and B (EHSVYILPP).

Fig. 3. Representative two-dimensional images of the SGE from *O. erraticus*. All the gels in this figure were 12% polyacrylamide. Reference molecular masses are indicated on the left. (A) Silver-stained gel with a 3-10 pH range. The non-antigenic spot No. 41 was cut from this gel and analyzed by mass spectrometry. (B) Sypro Ruby-stained gel with a 3-10 pH range showing the most abundant proteins in the SGE. (C and D) Silver-stained gels with 5-8 and 7-10 pH ranges showing the non-antigenic (circled and numbered in black) and the antigenic spots (circled and numbered in white) that were analyzed by mass spectrometry. (E and F) 2D-Western blots in the 5-8 and 7-10 pH ranges, showing the antigenic spots revealed by a pool of sera from pigs experimentally infested with *O. erraticus*. Antigenic spots are indicated by white numbered circles and are equivalent to the proteins identified in silver-stained gels in panels C and D. The white numbered circles indicate the antigenic spots localized in the homologous silver-stained gels displayed in panels C and D.

Figure 1

Fig. 1.

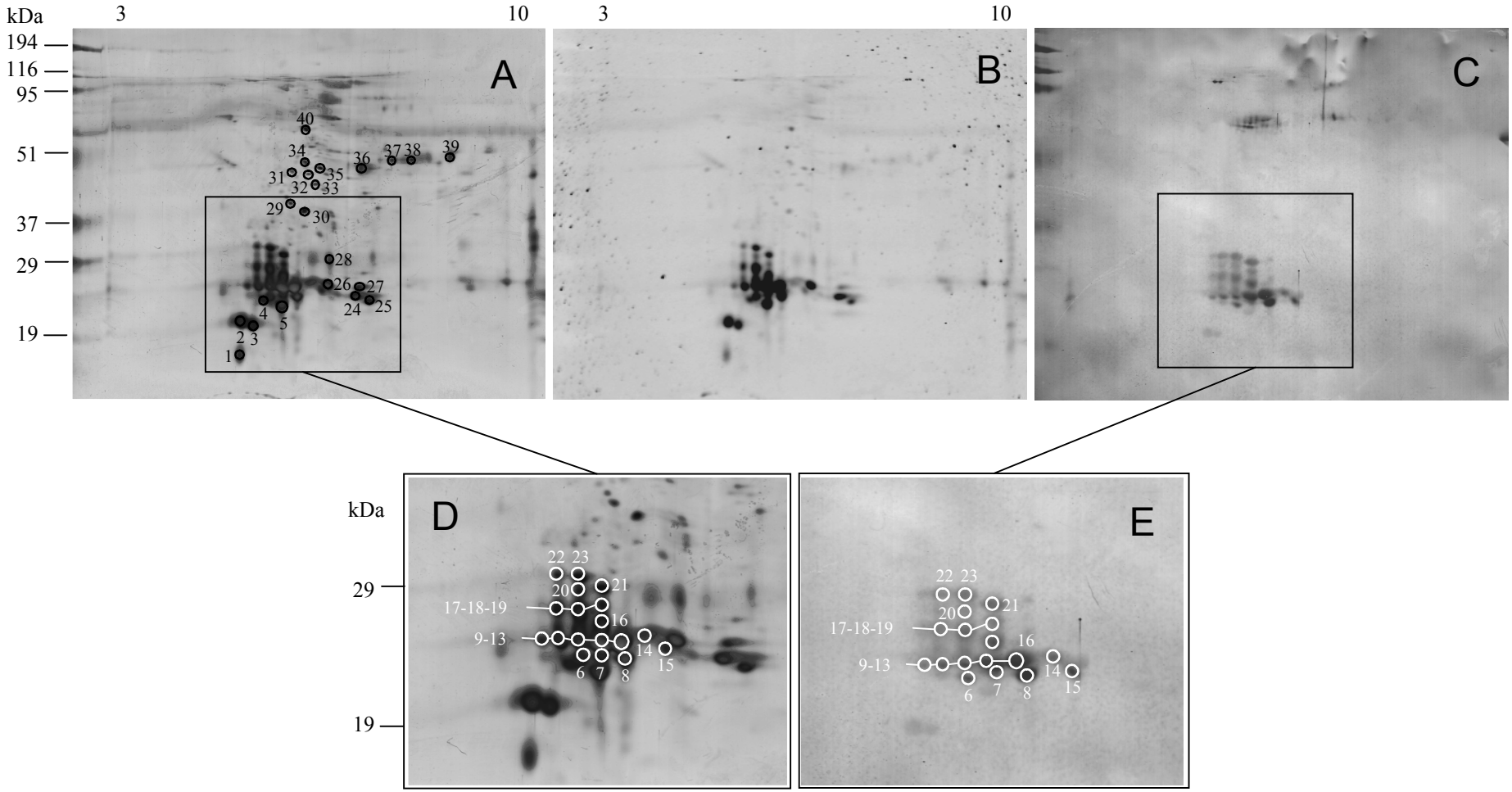
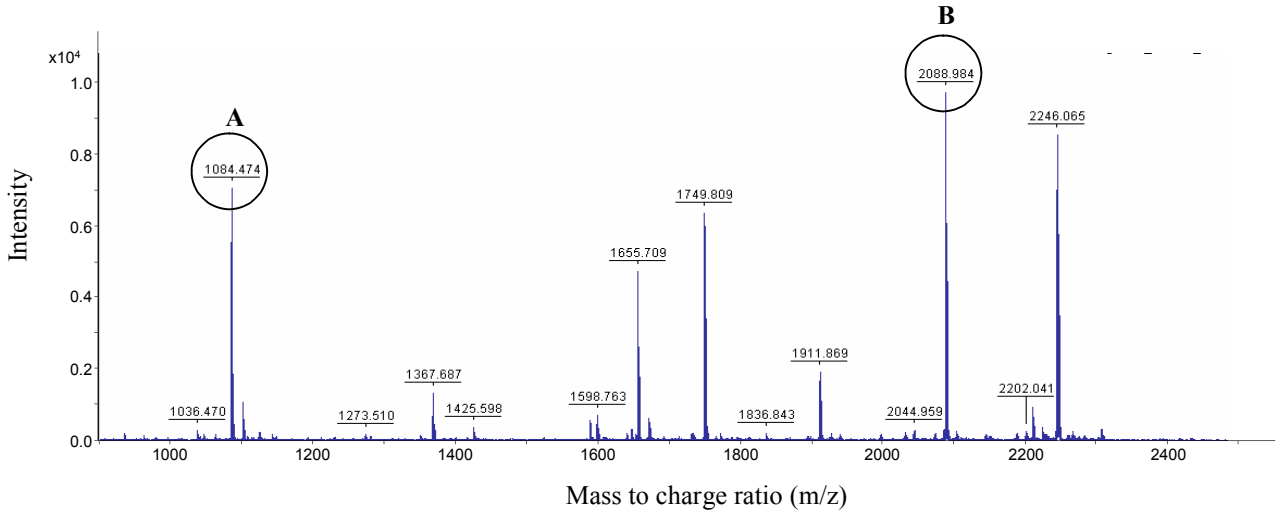


Figure 2


A
B
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0.6
0.4
0.2
0.0
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2246.065
1749.809
1655.709
1911.869
1367.687
1598.763
2202.041
1036.470
1273.510
1425.598
1836.843
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1400
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2000
2200
2400
Mass to charge ratio (m/z)

Figure 3

Fig. 3.

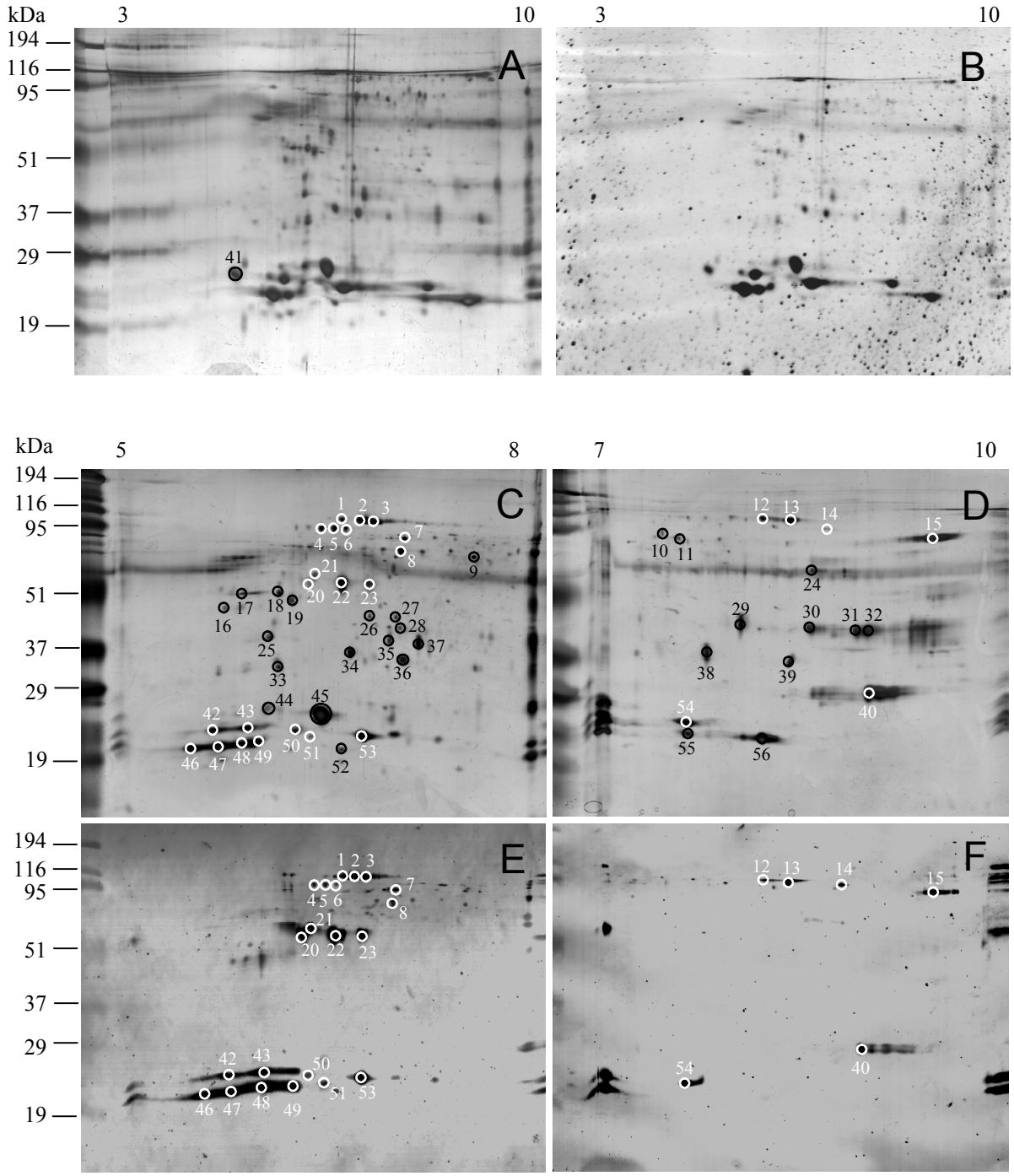

Table 1

Table 1. Summary of proteins identified in the SGE of *O. moubata* by MS/MS and *de novo* sequencing

Spot number	Antigenic	Protein ID[a] (Genbank accesión No.)	Peptide sequences[b]	% Sim. of peptide to protein	Homology to translated tick EST[c]	MW (kDa) Theo/exp[d]	pI Theo/exp[d]
6-23[e]	yes	TSGP1 *Ornithodoros savignyi* (AAN76828)	^{59}EHSCVYILPP^{68}	90	-	20.7/ 23.6-31.1	5.0/ 5.3-6.3
			^{79}YPYYMGYK^{86}	87.5	-		
4, 5[f]	no	Moubatin precursor *Ornithodoros moubata* (Q04669)	^{107}GELVYDVQSHDCHITK^{122}	100	-	18.8/ 22.6-23.3	5.7/ 5.5-5.8

[a]Protein in the NCBI database to which significant sequence similarity was observed.
[b]Sequence information obtained from *de novo* sequencing (spots No. 6-23) and from tandem mass spectrometry (spots No. 4 and 5). The regions of the protein to which the peptides align are noted by superscripts indicating residue number.
[c]Tick EST searched through The Gen Index databases (http://compbio.dfci.harvard.edu/tgi/): *Amblyoma variegatum*, *Boophilus microplus, Ixodes scapularis* and *Riphicephalus appendiculatus*.
[d] Theo/exp, theoretical predicted value/experimental observed value.
[e]Spots Nº 6-23 are isoforms of the same protein.
[f]Spots Nº 4 and 5 are isoforms of the same protein.

Table 2

Table 2. Summary of proteins identified in the SGE of *O. erraticus* by *de novo* sequencing based on MS/MS data.

Spot number	Antigenic	Protein ID[a] (Genbank accesión No.)	Peptide sequences[b]	% Sim. of peptide to protein	Homology to translated tick EST[c]	% Sim. of peptide to EST	MW (kDa) Theo/exp[d]	pI Theo/exp[d]
22	yes	NADPH dehydrogenase subunit 5 *Ornithodoros porcinus* (NP_996546)	^{225}LSMIFQIEMFTK^{236}	91.7	-	-	62.7/62.4	5.8/6.4
25	no	unknown secreted protein DS-1 precursor *Ornithodoros moubata* (AAS94229)	^{89}SHVVVDIIHGVITVHSK^{105}	100	-	-	38.8/41.1	5.2/6.0
41	no	Proteasome alpha subunit *Ornithodoros moubata* (AAS01024)	^{92}ARVEAQNHR^{100}	100	Proteasome subunit alpha type (EC3.4.25.1) *A. variegatum* (BM290335)	100	26.6/25.9	5.0/4.9
49	yes	ATP synthase F0 subunit 6 *Ornithodoros moubata* (NP_722566)	^{42}FQTIWFIISSMIVK^{55}	92.9	-	-	25.6/23.7	8.8/6.0

[a]Protein in the NCBI database to which significant sequence similarity was observed.
[b]Sequence information obtained from *de novo* sequencing based on MALDI-MS/MS data. The regions of the protein to which the peptides align are noted by superscripts indicating residue number.
[c]Tick EST searched through The Gen Index databases (http://compbio.dfci.harvard.edu/tgi/): *Amblyoma variegatum*, *Boophilus microplus, Ixodes scapularis* and *Riphicephalus appendiculatus*.
[d]Theo/exp, theoretical predicted value/experimental observed value.

Table 3

Table 3. Summary of proteins of the SGE of *O. erraticus* analysed by LC-MS/MS and *de novo* sequencing.

Spot number	Antigenic	Protein ID[a] (Genbank accesión No.)	Peptide sequences[b]	% Sim. of peptide to protein	Homology to translated tick EST[c]	% Sim. of peptide to EST	MW (kDa) Theo/exp[d]	pI Theo/exp[d]
45	no	n.i. [e]	DLQTECAYAK	-	-	-	-/26.8	-/6.4
47,48	yes	Lipocalin *Argas monolakensis* (ABI52694)	VREVGTGCGPY	-	-	-	18.7/ 22.7-23.9	7.2/ 5.5-5.7
			AEFLGSAGEK	-	-	-		
			NLDEQDDFGK	-	-	-		
			^{112}TTVLYSDYK^{120}	100	-	-		
53	yes	Alpha tubulin *Drosophila melanogaster* (NP_524297)	FNEELQK	-	-	-	50/24.8	5.2/6.6
			^{327}DVNAAIATIK^{336}	100	Alpha tubulin *A. variegatum* (BM290209), *I. scapularis* (G893P560FB9)	90		
			L^{443}LDEDEDY^{449}GK	85.7	-	-		
54	yes	n.i.	WYPTCGQNPL	-	-	-	-/23.8	-/7.7
			YPGAQQFLTVDQK	-	-	-		
			TQVLYSDYK	-	-	-		

[a]Protein in the NCBI database to which significant sequence similarity was observed.
[b]Sequence information obtained from *de novo* sequencing based on LC-MS/MS data. The regions of the protein to which the peptides align are noted by superscripts indicating residue number.
[c]Tick EST searched through The Gen Index databases (http://compbio.dfci.harvard.edu/tgi/): *Amblyoma variegatum*, *Boophilus microplus, Ixodes scapularis* and *Riphicephalus appendiculatus*.
[d]Theo/exp, theoretical predicted value/experimental observed value.
[e]n.i., not identified.
[f]Spots Nº 47 and 48 are isoforms of the same protein.