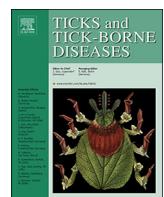




Ticks and Tick-borne Diseases

journal homepage: www.elsevier.com/locate/ttbdis



Original article

Evaluation of the protective efficacy of *Ornithodoros moubata* midgut membrane antigens selected using omics and *in silico* prediction algorithms

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ARTICLE INFO

Keywords:
Ornithodoros moubata
 Mialome
 Vaccines
 Midgut antigens
 Recombinant antigens
 Synthetic peptides

ABSTRACT

The African argasid tick *Ornithodoros moubata* transmits two important pathogens, the African swine fever virus and the spirochete *Borrelia duttoni*, the cause of human relapsing fever. To date, only conventional control measures such as widespread application of acaricides, strict control measures, and animal movement restrictions have been implemented to confine these diseases. Vaccines against tick infestations have the potential to be among the most efficacious interventions for the management of these diseases. Plasma membrane-associated proteins upregulated in tick midgut cells in response to blood feeding and digestion are thought to play vital functions in tick physiology and in the transmission of tick-borne pathogens. In addition, their antigenic extracellular regions are easily accessible to antibodies synthesised by immunised hosts, which makes them interesting targets for tick vaccine design. The mialomes (midgut transcriptomes and proteomes) of unfed *O. moubata* females and of engorged females at 48 h post-feeding have recently been obtained, providing a wealth of predicted midgut protein sequences. In the current study, these mialomes were screened using *in silico* tools to select predicted antigenic transmembrane proteins that were upregulated after feeding (516 proteins). The functionally annotatable proteins from this list (396 proteins) were then manually inspected following additional criteria in order to select a finite and easy-manageable number of candidate antigens for tick vaccine design. The extracellular antigenic regions of five of these candidates were obtained either as truncated recombinant proteins or as KLH-conjugated synthetic peptides, formulated in Freund's adjuvant, and individually administered to rabbits to assess their immunogenicity and protective potential against infestations by *O. moubata* and the Iberian species *Ornithodoros erraticus*. All candidates were highly immunogenic, but provided low protection against the *O. moubata* infestations (ranging from 7% to 39%). Interestingly, all candidates except one also protected against infestations by *O. erraticus*, achieving higher efficacies against this species (from 20% to 66%). According to their protective potential, three of the five antigens tested (Om17, Om86 and OM99) were considered little suitable for use in tick vaccines, while the other two (OM85 and OM03) were considered useful antigens for tick vaccine development, deserving further studies.

1. Introduction

The argasid tick *Ornithodoros moubata* colonises wild and anthropic habitats throughout South and East Africa, feeding on warthogs, domestic swine and humans (Vial, 2009). *O. moubata* transmits the African swine fever (ASF) virus and the human relapsing fever (TBRF) agent, *Borrelia duttoni*. The presence of this tick in anthropic environments contributes to the persistence of ASF and TBRF in endemic areas and may facilitate the spread of these diseases into surrounding areas (Cutler, 2010; Costard et al., 2013; EFSA panel, 2014; Sánchez-Vizcaíno et al., 2015; Quembo et al., 2016). It is expected that elimination of synanthropic populations of *O. moubata* would greatly

improve the prevention and control of such diseases.

Anti-tick vaccines have emerged as a cost-effective and environmentally sustainable method for the control of tick infestations and tick-borne diseases (Willadsen, 2008; de la Fuente et al., 2016; Šmit and Postma, 2016).

Our team has made substantial efforts in the development of an anti-*Ornithodoros* vaccine, testing the protective effects of two types of antigens: salivary antigens, which are naturally exposed to the host immune system during tick feeding, and concealed antigens from the tick midgut (Astigarraga et al., 1995; Díaz-Martín et al., 2015a).

The studies with salivary antigens resulted in the identification of three anti-haemostatic proteins that displayed individual vaccine

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efficacies between 27% and 44%, and up to 60% efficacy when they were administered together as a multicomponent vaccine, making them interesting vaccine candidates. The main protective effect of these antigens consisted of the partial inhibition of blood feeding and subsequent reduction in female fertility, most likely as a consequence of the antibody-mediated loss of function of the target antigen at the tick-host interface. These vaccine-induced antibodies were mainly directed to immune-dominant linear B-cell epitopes located on the surface of the target salivary antigens (Díaz-Martín et al., 2015b). Despite these promising results, a fully protective vaccine against *O. moubata* based only on salivary antigens has not been obtained, which makes it necessary to continue searching for new and more protective antigens in other tick tissues and organs (García-Varas et al., 2010; Díaz-Martín et al., 2015a,b).

O. moubata midgut antigens seem very promising as vaccine candidates because a midgut antigen, the Bm86 protein, is the basis of the only two tick vaccines marketed to date, TickGARD® and GAVAC®. The Bm86 antigen induces protective immune responses mainly mediated by host complement system and antibody interactions which damage the tick midgut wall subsequently disrupting tick survival and egg viability (de la Fuente et al., 2007; Lew-Tabor and Rodriguez Valle, 2016).

Not surprisingly, animal vaccination with crude extracts of midgut membranes from *O. moubata* — and with similar extracts from the Iberian species *Ornithodoros erraticus* — induced protective responses that reduced female feeding and fertility by up to 50% in both species and, additionally, caused up to 80% mortality to the nymphs of *O. erraticus* in the first 72 h post-feeding.

The antigens responsible for this protection were partially characterised as membrane proteins of the luminal surface of midgut epithelial cells (enterocytes), whose expression increased upon blood ingestion, peaking between 24 and 72 h post-feeding (h.p.f.). The protective immune mechanism involved the fixation and activation of the host complement system onto the membrane of enterocytes by specific vaccine-induced antibodies. This caused enterocyte lysis and midgut damage in a similar way to what was observed for the vaccines based on the Bm86 antigen (García-Varas, 2004; Manzano-Román et al., 2006, 2007).

The identity of these proteins remains unknown, but the above results confirmed the protective potential of midgut antigens from both *Ornithodoros* species, establishing this organ as a suitable source of antigens for soft tick vaccine development.

Blood digestion and the absorption of the released nutrients take place in the tick midgut; moreover, the midgut constitutes a pivotal entry point for tick-borne pathogens that determines the pathogen success in survival, vector colonisation and subsequent transmission (Narasimhan et al., 2014; Abraham et al., 2017). Accordingly, the tick midgut expresses a wide range of proteins that play vital functions in digestion-related physiological processes and in the infection and transmission of blood-borne pathogens (Kocan et al., 2004; Sojka et al., 2013, 2016). Many of these proteins are upregulated in response to the stimulus provided by host binding and blood ingestion (Sojka et al., 2013; Oleaga et al., 2015; Sojka et al., 2016; Oleaga et al., 2017a,b). According to previous observations, it may be expected that some of the midgut proteins that are upregulated upon tick feeding have protective potential (Akov, 1982; Matsuo et al., 2003; García-Varas, 2004; Manzano-Román et al., 2006, 2007); in other words, that the disruption of their function by vaccine-induced antibodies might have a significant impact on tick physiology and survival.

Among midgut proteins, transmembrane proteins expressed on the luminal side of the enterocyte plasma membrane are interesting candidates in vaccine design because their antigenic extracellular regions are easily accessible to host immune effectors — mainly antibodies — ingested in blood (Rappuoli and Bagnoli, 2011). Hence, midgut transmembrane proteins are considered first-election targets for the development of new drugs and vaccines aimed at tick control (Richards et al.,

2015).

Recently, our team obtained the mialomes (midgut proteomes and transcriptomes) of *O. moubata* female ticks in two physiological conditions: before feeding and in the initial phases of the blood digestion, at 48 h.p.f. These studies have provided an unprecedented number of novel nucleotide sequences coding for midgut proteins from *Ornithodoros* ticks, further allowing the identification of genes/proteins that are differentially expressed upon blood feeding (Oleaga et al., 2017a,b).

These data can be screened with suitable bioinformatics tools to identify predicted proteins with particular traits in cellular localisation, topology, antigenicity and biological function in order to select potential antigenic candidates for vaccine development. The selected candidates can then be produced, mainly as recombinant proteins, and their protective efficacy experimentally tested in animal immunisation trials (Richards et al., 2015; Lew-Tabor and Rodriguez Valle, 2016; de la Fuente et al., 2016).

Accordingly, the aim of the current work was to identify new protective antigens from the *O. moubata* mialome, with the following specific objectives: first, the *in silico* selection of potentially protective candidate antigens of *O. moubata* among transmembrane proteins up-regulated in the midgut after feeding; second, the production of these candidates as recombinant proteins or synthetic peptides; and, finally, their experimental assessment and validation as vaccine antigens in animal immunisation trials.

2. Materials and methods

2.1. Ticks and tick material

The *O. moubata* and *O. erraticus* ticks used in this study were all obtained from the two colonies currently maintained at the laboratory of Animal Parasitology, IRNASA, CSIC, Spain. The colony of *O. moubata* was established from specimens obtained from the Institute for Animal Health in Pirbright (Surrey, UK) and the colony of *O. erraticus* was established from specimens captured in Salamanca, western Spain. These ticks are regularly fed on rabbits and kept in a culture chamber at 28 °C, at 85% relative humidity with a 12 h light-dark cycle. Tick feeding and animal manipulation was performed according to the regulations established by the Ethical and Animal Welfare Committee of the institution where the experiments were conducted (IRNASA, CSIC), following the corresponding EU legislation (Directive 2010/63/EU).

Midguts from engorged *O. moubata* females at 48 h.p.f. were obtained as described by Oleaga et al. (2017a) and preserved in RNA-later (Ambion) for RNA extraction. Total RNA was extracted and purified using the RNeasy Mini Kit (Qiagen) following the manufacturer's instructions.

Midguts from unfed *O. moubata* and *O. erraticus* females and from engorged females at 48 h.p.f. were also obtained and used to prepare extracts of soluble and membrane proteins as previously described (Oleaga et al., 2017b). Briefly, batches of 25 midguts from each species and physiological condition were homogenised and sonicated in ice-cold PBS containing proteinase inhibitors (Roche Diagnostics, Indianapolis, USA). Tissue homogenates were centrifuged at 10⁴ g to remove particulate remnants, and the supernatants were fractionated by centrifugation at 10⁵ g into two fractions enriched in either soluble or membrane proteins (the 10⁵ g supernatants and pellets, respectively). The protein concentrations in these samples were assessed using the BCA Protein Assay Reagent kit (Thermo-Fisher, Rockford, USA), and the samples were stored at –20 °C.

Tick saliva from unfed female *O. moubata* and *O. erraticus* ticks was collected after stimulating them with 1% pilocarpine, as described previously (Díaz-Martín et al., 2013). Protein concentrations in the saliva samples were measured using the Bradford assay (Bio-Rad) and samples were stored at –20 °C.

2.2. In silico prediction of upregulated antigenic transmembrane proteins in the *O. moubata* midgut

Gene expression data from the midgut of *O. moubata* females before feeding (unfed, basal condition) and at 48 h.p.f. (fed), as well as a differential gene expression analysis between these two physiological conditions, were obtained from a previous transcriptomic study by Oleaga et al. (2017a). The resulting Transcriptome Shotgun Assembly (TSA) project, including the nucleotide and protein sequences, was deposited in the DDBJ/EMBL/GenBank database under the accession GFJQ00000000 (BioProject PRJNA377416).

Briefly, gene transcription levels were estimated using the RPKM method (Mortazavi et al., 2008) for both conditions, and level comparisons between fed and unfed females were performed using the DEseq algorithm (Love et al., 2014), considering a false discovery rate (FDR) limit of 0.05 (FDR < 0.05). Genes showing a fold change > 2 in fed female ticks were considered differentially upregulated.

The putative open reading frames present in the upregulated gene transcripts were conceptually translated to amino acid sequences using the Transdecoder software (<https://transdecoder.github.io/>) and the resulting proteins were analysed in the following way.

First, they were screened using the VaxiJen 2.0 software to determine the likelihood of the predicted proteins to be putative antigens using the 0.5 antigenicity threshold established by default for endoparasitic organisms in the VaxiJen website (<http://www.ddg-pharmfac.net/vaxijen/vaxijen/vaxijen.html>) (Doytchinova and Flower, 2007a,b).

Next, for the predicted antigenic proteins, the number of putative signal peptides and transmembrane helices, which would indicate protein secretion or membrane location, were determined using the SignalP-4.1 (<http://www.cbs.dtu.dk/services/SignalP/>) (Petersen et al., 2011) and TMHMM (<http://www.cbs.dtu.dk/services/TMHMM/>) servers, respectively. In the output list, proteins lacking signal peptides and transmembrane regions were considered intracellular; proteins with signal peptides but without transmembrane regions were considered secretory; and proteins having one transmembrane region without a signal peptide or at least two transmembrane regions if a signal peptide was present were considered to be transmembrane proteins.

2.3. Functional annotation, manual inspection and selection of antigenic candidates

The final list of predicted antigenic and upregulated transmembrane proteins was functionally annotated based on BLAST searches (E-value < 10⁻⁰⁵) against sequences in the NCBI non-redundant sequence database (<http://www.ncbi.nlm.nih.gov/>), the Swiss-Prot database (<http://www.uniprot.org/>) and the non-redundant genome-wide sequence database for *Ixodes scapularis* (http://metazoa.ensembl.org/Ixodes_scapularis/Info/Index).

Transcripts/proteins that could not be annotated were excluded from the ensuing analyses in the present work. The annotated proteins (Supplementary Table 1) were manually inspected in order to filter and rank them and facilitate selection of a limited number (< 10) of potential antigenic candidates to be produced as recombinant proteins – or synthetic peptides- and their vaccine efficacy tested in animal trials.

To this end, proteins with incomplete sequences – those showing stretches of unresolved nucleotide positions- were excluded from further analyses; multi-spanning proteins with more than four transmembrane domains were also excluded because proteins with multiple transmembrane regions are more difficult to express and purify (Pizza et al., 2000); and proteins expressed in internal membranes were also excluded because intracellular proteins are less accessible as antigenic targets for antibodies (Rappuoli and Bagnoli, 2011).

The remaining proteins were ranked according to their expression

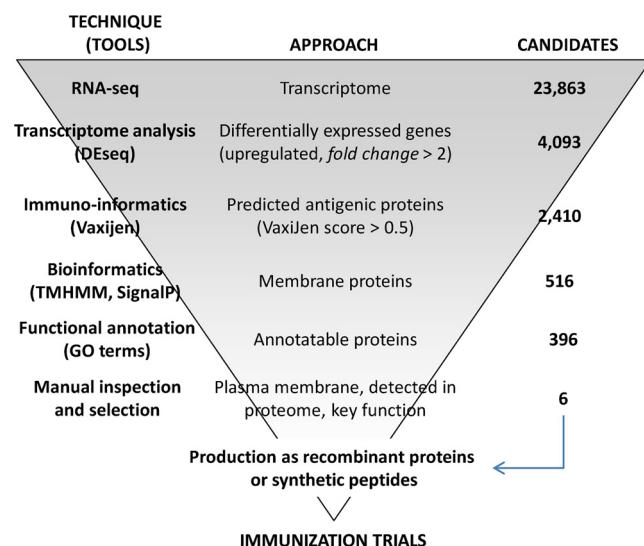


Fig. 1. The bioinformatics-based approach applied to identify potential vaccine candidates from the *Ornithodoros moubata* midgut transcriptome. Systematic *in silico* analysis of transcriptomic data was followed by manual inspection for the selection of candidate antigens with the desired traits was used. In this way, among the 396 upregulated transcripts annotated as antigenic membrane proteins, multi spanning proteins with more than four transmembrane domains, proteins expressed on intracellular membranes and proteins with incomplete sequences were excluded. The remaining proteins/transcripts were ranked according to their expression level, expression increase in fed ticks and predicted antigenicity. The top 30 proteins in each rank were selected and combined giving a list of 75 unique proteins. Among them, priority for selection was given to proteins expressed on the plasma membrane, to proteins detected in the midgut proteome and to proteins with key functions in blood digestion. Finally, six of these proteins were selected as antigenic candidates, produced as recombinant proteins or synthetic peptides and their vaccine efficacy evaluated in rabbit vaccine trials.

level, expression increase in fed ticks and predicted antigenicity (VaxiJen score). The top 30 proteins in each rank were selected and combined to obtain a list of non-redundant proteins (i.e., the repeated proteins were recorded only once). The proteins in this list were then manually inspected in order to select a limited number of antigenic candidates.

In this selection, priority was given to single-spanning proteins over multi-spanning proteins, to proteins predicted to be associated with the plasma membrane and to proteins/transcripts predicted to have important functions in tick physiology, particularly to proteins involved in processes related to blood digestion, nutrient transport and metabolism, haem and iron storage and management, defence responses, and responses to oxidative stress (Oleaga et al., 2017a,b). Finally, the presence of the predicted proteins in the *O. moubata* midgut proteome (Oleaga et al., 2017b) was also scored, although no candidates of interest were discarded for not being detected in the proteome.

According to these criteria, six theoretical candidates were chosen to be experimentally produced and evaluated in animal immunisation trials (Fig. 1, Table 1).

2.4. Analysis of the topology of the candidates and selection of extracellular regions to be produced as recombinant proteins or synthetic peptides

The full-length cDNA coding sequences of the selected candidates had already been amplified by RT-PCR and cloned into the pSC-A sequencing vector (Agilent) by Oleaga et al. (2017a), in order to verify their assembly (Supplementary Table 2).

The deduced amino acid sequences of the seven candidates were analysed in depth using the TMHMM and SACS TMHMM (<http://www.sacs.ucsf.edu/cgi-bin/tmhmm.py>) tools (Krogh et al., 2001; Moller

Table 1

Candidates selected to be produced and evaluated as protective antigens in animal vaccination trials. TM, number of transmembrane domains; PM, expression in the plasma membrane; PROT, found in the midgut proteome.

Candidate	Uniprot ID. Protein name	Molecular function	Expression level in fed ticks	Fold change	VaxiJen score	TM	PM	PROT
Om99	Q4PM45. Protein NEF1, putative	protein N-linked glycosylation	9463,61	14.4	0.8195	2	–	–
Om86	E5E7I8. Os86 (Bm86 homologue)	unknown	17672,63	1	0,6533	1	yes	yes
Om85	B7P5F1. Transmembrane protein, putative	unknown	329,83	5.0	0.7846	3	–	yes
Om29	B7Q6A8Tetraspanin. TM4SF family	cell surface receptor signalling pathway	18476,10	2.9	0.6129	4	yes	yes
Om28	B7Q9D8. Organic cation/carnitine transporter, putative	transmembrane transporter activity	219,71	216	0.5535	1	yes	yes
Om17	H2XVL1. Receptor expression-enhancing protein.	transmembrane transporter activity	757,78	2.05	0.8514	2	yes	–
Om03	B7PMP9. Conserved hypothetical protein	unknown	406,48	2.1	1.0043	1	yes	–

et al., 2001) in order to establish their topology and define their extracellular regions. Additionally, the presence of signal peptides was confirmed using the SignalP-4.1 server, the absence of non-classical secretion signals was detected by the SecretomeP 2.0 server (<http://www.cbs.dtu.dk/services/SecretomeP/>; Bendtsen et al., 2004), and the absence of GPI anchors was detected using the GPI-SOM server (<http://gpi.unibe.ch/>; Fankhauser and Maser, 2005).

In this way, the candidates with extracellular regions longer than 50 amino acids (Om86, Om29, Om28 and Om17) were chosen for cloning and expression of their extracellular regions as recombinant proteins (see below). Candidates whose extracellular domains were shorter than 50 amino acids (Om99, Om85 and Om03) were inspected for the presence of linear B-cell epitopes on such extracellular domains, in order to use the predicted B-cell epitope sequences for the design of synthetic immunogenic peptides.

Linear B-cell epitope predictions were performed using three immunoinformatical tools: ABCpred (<http://www.imtech.res.in/raghava/abcpred/index.html>) (Saha and Raghava, 2006), BCEpred (<http://www.imtech.res.in/raghava/bcepred/>) (Saha and Raghava, 2004), and BepiPred-2.0 (<http://www.cbs.dtu.dk/services/BepiPred/>) (Larsen et al., 2006). Amino acids predicted to form part of a B-cell epitope by at least two of these tools were included in the sequence of the immunogenic peptides, which were designed to be between 16 and 18 amino acids long.

The peptides were synthesised and coupled to the carrier protein KLH via an added N-terminal cysteine residue at JPT Peptide Technologies GmbH (Berlin, Germany). These peptides were used as immunogens in rabbits. The same peptides were also synthesised without KLH conjugation and used as coating antigens in ELISA tests for the analysis of the antibody response in the immunised rabbits. To increase adsorption to the ELISA plates, the peptides were allowed to form oligomers. To this end, a cysteine residue was added at each end of the peptides to allow oligomerisation through spontaneous oxidation of the cysteine sulphhydryl groups (Caro-Aguilar et al., 2005). These unconjugated peptides were synthesised at Biomedal (Sevilla, Spain).

2.5. Subcloning and expression of the extracellular regions of the candidates Om86, Om29, Om28 and Om17

cDNA fragments coding for the extracellular regions of candidate proteins (rtOm86, rtOm29, rtOm28 and rtOm17) were amplified by PCR from the corresponding full length cDNAs cloned in the pSC-A vector. For these amplifications, four specific primer pairs were designed that included suitable restriction sites to assist in the sub-cloning into the pQE-30 expression vector (Qiagen). Supplementary Table 3 shows the primers and conditions used for these amplifications. The PCR products were purified, digested and cloned into the pQE-30 vector following standard procedures described previously (Díaz-Martín et al., 2011).

Recombinant pQE-30 plasmids containing the cDNA fragments rtOm86, rtOm29, rtOm28 and rtOm17 were transformed into *E. coli*

M15 cells (Qiagen) and protein expression was induced with 1 mM IPTG following standard procedures.

The recombinant rtOm86 and rtOm17 proteins were readily expressed, although the protein products were insoluble. By contrast, the expression of the recombinant rtOm29 and rtOm28 proteins failed.

2.6. Solubilisation and purification of rtOm86 and rtOm17

The recombinant rtOm17 protein was solubilised in 10% sarcosyl in lysis buffer according to the protocol by Massiah et al. (2016). The solution was then diluted five times with lysis buffer resulting in a sarcosyl concentration of 2%, compatible with nickel affinity chromatography (de Marco, 2007). rtOm17 was then purified by nickel affinity chromatography according to the procedure described by Díaz-Martín et al. (2011).

The recombinant rtOm86 protein was not solubilised in 10% sarcosyl or using increasingly harsh solubilising agents such as 8 M urea, 8 M urea + 20 mM 2-mercaptoethanol or 8 M urea + 5 mM DTT (Qiagen, 2003). Therefore, the recombinant protein was purified from the cellular lysate pellet by electro-elution from SDS-PAGE gels following the procedure described by Harlow and Lane (1988).

The concentrations of the purified proteins were measured by densitometry in Coomassie blue-stained SDS-PAGE gels followed by interpolation into a bovine serum albumin (BSA) standard curve. Purified proteins were stored at –20 °C.

2.7. Vaccine trials

2.7.1. Trial 1

The aim of this trial was to assess the protective potential of the rtOm86 and rtOm17 candidate antigens against infestations by *O. moubata* and *O. erraticus*. Each recombinant protein was administered to a group of three rabbits (New Zealand white) in Freund's adjuvant. An additional control group of rabbits was treated with the adjuvant alone. Each animal was vaccinated with three doses at 15-day intervals, each comprising 100 µg of protein, administered subcutaneously. The first dose was administered emulsified in Freund's complete adjuvant (FCA) and the second and third doses in Freund's incomplete adjuvant (FIA).

Rabbits were bled immediately before administration of the first dose (pre-immune sera) and at 14 and 28 days after the third dose (immune sera), immediately before tick infestations. Blood samples were allowed to clot and sera were removed and stored at –80 °C.

In the immune sera, the IgG antibody titres to the recombinant proteins were measured by ELISA according to standard procedures (García-Varas et al., 2010). Briefly, polystyrene plates (Sigma) were coated with 100 ng of recombinant antigen per well in 100 µl of carbonate buffer, pH 9.6, at 4 °C overnight and post-coated with 1% bovine serum albumin in PBS. The sera were diluted in TPBS (PBS containing 0.05% Tween 20) in two-fold dilution series starting at 1/100, and each dilution was analysed in duplicate wells. Peroxidase-conjugated anti-

Table 2

Amino acid sequences of the proteins Om99, Om86, Om85, Om29, Om28, Om17 and Om03, selected as antigenic candidates. Indicated are their lengths in amino acids (aa), isoelectric points (*pI*), molecular weights (MW) in kDa, and the presence of signal peptide (Signal P) and the number of the transmembrane domains (TM). Numbers in brackets indicate the amino acid positions occupied by either the Signal P or the TMs. The only Signal P (in Om86) is highlighted in bold, and the transmembrane domains (TM) are marked in bold and underlined.

Candidate	Sequence	aa	<i>pI</i>	MW	Signal P (aa)	Number of TM (aa)
Om99	1 MSFNSVGLGLSIEQMHKYVSPVNP A VYPH <u>LTLVLMGIGLFFMAWFFIYEV</u> TSTKFRDIF 61 KELLISLVAAVFLGFGVLFLLLWVGIVV	88	6.7	9.9	No	2 (25–47, 64–86)
Om86	1 MCSLRIVLVLSVLRNITAASVSDEQQGEVVRPSQDVCRVGNALCGSHTCVAQPNEG 61 FFCDCGQDHFYDVRAKCTHIQSCTASRCQFGICSDEGRTVPATCDCTDIANLTPSECVT 121 PEKKECSSIGAVPSVGPNSEVTCRCLPPTCLCRPEVKNDRCLPTCLNFSNTCEQLCKRKQLINE 181 DHRCQCQWHDVCTAPEVEGGSCPEPGYIKATSQDGCIACSGETNPVCPGSGTSNTTDG 241 MPYNCVCGKDQELAHGDLGCLPKTGCSPEEKSCKREDQECVLEEGHVSACKCPANQLEIN 301 GQCSGNCTRECOHRFASCRISSDNQEQQCQCSPLEASSTGRDGSCVLEKYAYLTSPFKLNR 361 SLASWKVVYDCRQMKDSLMKAFLVFGSQFLTLIDITCKELYRVRLTFSKQDQAVLNRL 421 HLCKNSMKPETYFWPDVHVSGVGPVEAENICETALREPPIEKYEGGYVCKVEDDQVRF 481 NCVNPAAVSGTETSGRIJKQRCSSEENKLPGKGENGKDDEDQMPPNQT A TAVVGAVLAILA 541 ALGHIAIIIV SRKRKAARAEQRREKYEADLQQ	572	5.3	62.6	Yes (1–19)	1 (528–550)
Om85	1 MDLRDSEYRPLFGGPVDRPLFGGSNGRERTV <u>HMTLGIITTVLVSITVILAFFYSVNPPCG</u> 61 RHIYFALCIVALCVSHIILH WYRQGDVDPKFR <u>TLIYVNNTVVIILFCISALTYFVNRC</u>	118	8.4	13.7	No	3 (33–55, 62–84, 94–116)
Om29	1 MDDGIACVKVYLIACNLLVWILGLAVL SIGIWI RSPDPDFWIYQDNLPLSNYYDACYIIMA 61 AGVILLILGF MGCCAAAIDSPCMILTYFIAMLVLLM ECAVAGLV WKVADGDTLQRHLAT 121 TITAKIDEINDNPKARRFMIDLQMVHLECCGAISKHDYEVAMTIPOQSCSSRTNNIFIYIG 181 CSENLRVLLERTGAVV GGMGLALGFVQVIVMISLCLFCTIRQDKS	226	5.1	24.9	No	4 (13–35, 55–77, 84–106, 196–218)
Om28	1 MLEEAAMSEVGE <u>FGLFQYLLICYL</u> LVVFVAPLRLPFAHIFSFLVPPHRCRLPPHLDIA 61 SNTTTESLNLYIPLSDEGDSLHCKMYLHNSTIEDWSNNNSFTTSCLYGWEYDLSFY 121 PTIVSEVSSLTRCTYCVDTLRCCSVARYTRCCIRKR	156	5.9	18.1	No	1 (13–35)
Om17	1 MVSVI LSRLVLLFGTLYPAYASYKAVKTKNVREYVWMMYWIVFALFTCAETFADLLS 61 FWFPFYYEIKVLFVLWLSPATKGSSILYRKLVHPQLRR EEIDQFLLKARDQGYTAVV 121 QLGSKGLSYAAGVVMQTA ^{LRGHESLATHL} QKALPDEPRARSKKTESDTSSESSKSHGE 181 GPRRSSRRKVAKDRAKDS	198	9.8	22.6	No	2 (4–21, 41–63)
Om03	1 MVVDIRAWFEYVVEAAKSPGEFITYV VILLALSPFFLVS AFLSWKLAHKLEAEKGRKQRS 61 KKKNIAETRRTKSD	75	9.8	8.7	No	1 (24–46)

rabbit IgG (Sigma) was used diluted 1/10,000 in TPBS. Ortho-phenylene-diamine (OPD) was used as chromogen substrate for peroxidase and the reactions were stopped with 3N sulphuric acid. Incubations were performed at 37 °C for 1 h, and washes with TPBS were carried out at room temperature for 10 min per wash. The serum titre was defined as the highest dilution giving more than twice the reactivity of the corresponding pre-immune serum at the same dilution.

After titration, the reactivity of the immune sera to the saliva and the four midgut protein extracts (soluble and membrane proteins from fed and unfed females) from each species (*O. moubata* and *O. erraticus*) were tested by ELISA and western blot following standard procedures with minor modifications (Manzano-Román et al., 2015). The ELISA plates were coated with 1 µg of saliva or midgut extract per well in 100 ml of carbonate buffer, pH 9.6, at 4 °C overnight and post-coated with 1% bovine serum albumin in PBS. In the case of midgut membrane extracts, they were sonicated (3 times, 30 s. each) before and after diluting them in carbonate buffer to favour their homogeneous suspension and their binding to the plate. The sera were used diluted 1/300 in TPBS and the PO-anti-rabbit IgG diluted 1/10,000.

At 14 and 28 days after the third antigen dose, all rabbits received two tick infestations with 15 females, 25 males and 50 nymphs-3 of *O. moubata*, and 15 females, 25 males and 50 nymphs-3 of *O. erraticus* per rabbit and per infestation.

The parasites were allowed to feed on the rabbits for a maximum of 2 h. Usually, this time is long enough for all these tick species and developmental stages to complete engorgement and detach themselves. After 2 h, any tick still remaining on the animal was removed. The degree of protection was determined by measuring: (i) the amount of blood ingested, (ii) the female oviposition and fertility rates (that is, the number of eggs laid per female and the number of newly hatched nymphs-1/larvae per female), (iii) the moulting rate of nymphs-3, and (iv) the mortality rates of all developmental stages tested.

The values obtained for the parasites fed on the animals from each group were summarised as means ± standard deviations. Statistical differences between the vaccinated and control group were assessed by one-way ANOVA followed by Dunnett's *t*-test. Values of *p* < 0.05 were considered significant.

Additionally, the vaccine efficacy (E) for each recombinant antigen was calculated according to the formula established by Canales et al. (1997), and recently updated by Aguirre et al. (2015) and Contreras and de la Fuente (2016), which is based on the reduction in the studied developmental processes in ticks fed on vaccinated animals as compared to ticks fed on controls. Here, vaccine efficacy was calculated as E = 100 x [1 - (S x F)], where S and F respectively represent the reduction in female survival and fertility in ticks fed on vaccinated rabbits respect to those fed on control rabbits (Supplementary Table 4).

2.7.2. Trial 2

The aim of this trial was to evaluate the protective potential of the KLH-conjugated synthetic peptides OM99, OM85 and OM03 against infestations by *O. moubata* and *O. erraticus* ticks.

Three groups of three rabbits per group were given three doses of 100 µg of the corresponding KLH-conjugated peptide formulated in FCA (first dose) or FIA (second and third doses). A fourth control group of three rabbits was treated with 100 µg/dose of the KLH carrier protein formulated in Freund's adjuvant. These doses were chosen according to our former experience with peptide vaccines (Manzano-Román et al., 2015). As in the previous trial, the rabbits were bled immediately before the administration of the first dose (pre-immune sera) and at 14 and 28 days after the third dose, immediately before tick infestations (immune sera).

In the immune sera, the antibody titres to the homologous peptide and their reactivity to the other peptides were tested by ELISA. Moreover, the reactivity and specificity of the immune sera to the saliva

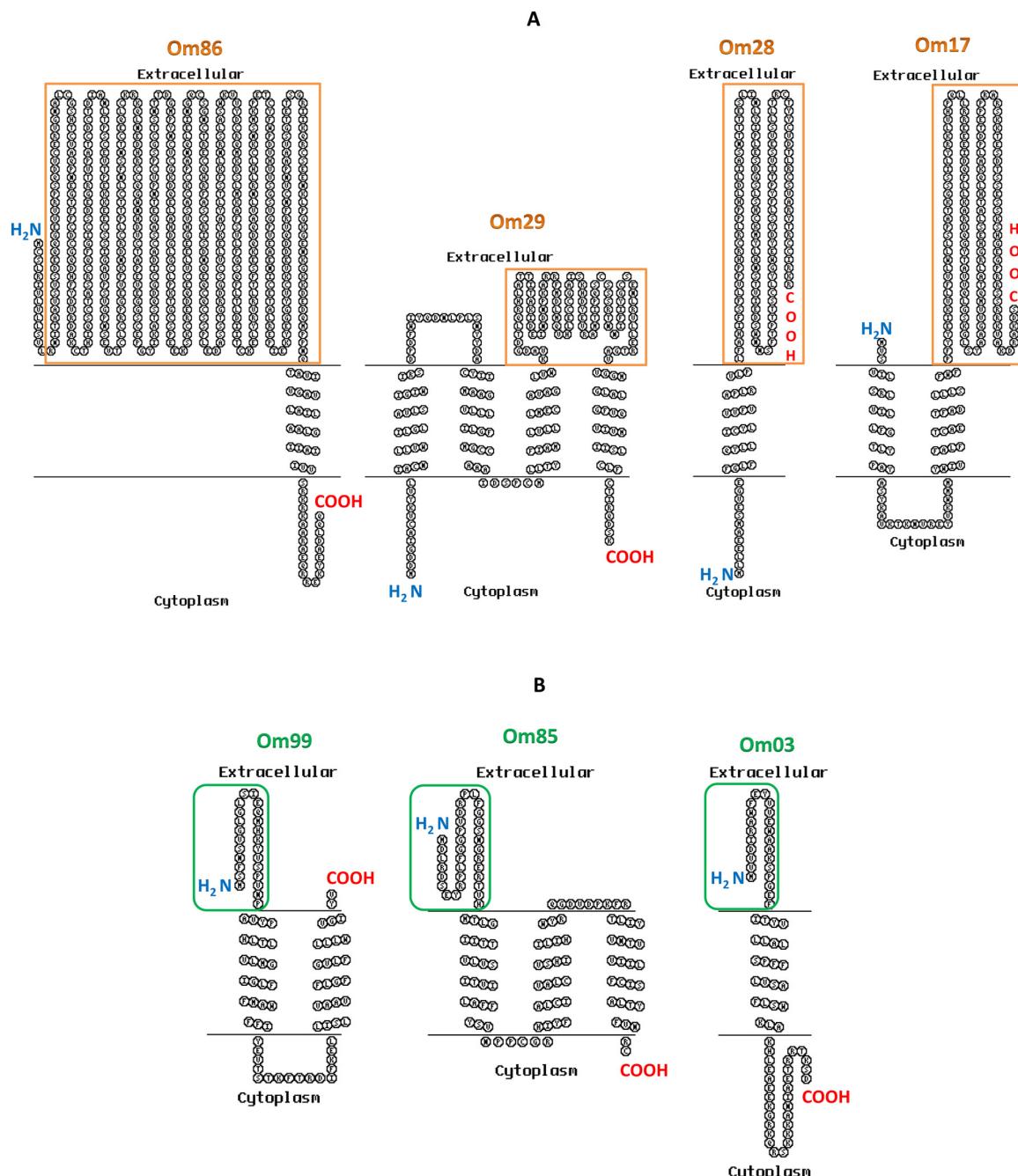


Fig. 2. Topology predictions for the seven candidates. (A) Most of the sequence of proteins Om86, Om29, Om28 and Om17 is extracellular or has at least one extracellular domain of more than 100 amino acids (orange squares). (B) For proteins Om99, Om85 and Om03, most of the sequence is transmembrane or intracellular, with only a short exposed extracellular amino-terminal domain of 24–32 amino acids (green boxes). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of both *O. moubata* and *O. erraticus* and to the four midgut protein extracts from each species were tested by ELISA and western blot in a similar way to that described above for trial 1.

At 14 and 28 days after the third antigen dose, all rabbits received two tick infestations comprising 15 females, 25 males and 50 nymphs-3 of *O. moubata*, and 15 females, 25 males and 50 nymphs-3 of *O. erraticus* per rabbit and per infestation.

The tick infestations, the assessment of vaccine effects on the parasites, and the calculation of the peptide vaccine efficacies were performed as described for trial 1 above.

3. Results and discussion

3.1. Candidates selected by *in silico* screening and manual inspection

In our previous study, 23,863 transcripts from the *O. moubata* female midgut were assembled (NCBI BioProject: PRJNA377416) (Oleaga et al., 2017a). Among these transcripts, 4093 were significantly upregulated in fed ticks, and up to 2410 of them code for antigenic proteins according to the VaxiJen predictions.

VaxiJen predicts antigens using an alignment-independent method that is based on the physicochemical properties of the protein amino acid sequences, which makes this tool suitable for antigen prediction in

Om99 (88 aa)

Om85 (118 aa)

Om03 (75 aa)

Fig. 3. Linear B-cell epitope predictions for the extracellular domain of the Om99, Om85 and Om03 proteins, and the designed synthetic peptides. The sequence of each protein is represented in triplicate, showing the ABCpred (yellow), BCEpred (blue) and BepiPred-2.0 (green) predictions. Amino acids with antigenicity predictions made by at least two of these three algorithms were included in the immunogenic peptide design (boxed). The predicted topology is indicated below each protein sequence: o (outside), extracellular; M, transmembrane; i, intracellular. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3

Names and sequences of the synthetic peptides designed from the extracellular domains of proteins Om99, Om85 y Om03. The design includes either an amino-terminal cysteine to facilitate conjugation to KLH or one cysteine in each peptide to allow spontaneous lineal oligomerization of the peptides. Added cysteines are underlined.

Name	No. of aa (without cysteines)	Sequences for synthesis and conjugation to KLH	Sequences for synthesis and lineal polymerization
OM99	16	KLH-CGLSIEQMHKYVSPVNP	CGLSIEQMHKYVSPVNP
OM85	16	KLH-CGGPVDRPLFGGSNGRE	CGGPVDRPLFGGSNGREC
OM03	18	KLH-CIRAWFEYVVWEAKSPGE	CIRAWFEYVVWEAKSPGEC

organisms whose genomes are poorly or not sequenced. VaxiJen has shown up to 70–89% prediction accuracy for bacterial, viral and tumour antigens and up to 78–97% accuracy for endoparasitic and fungal antigens (Doytchinova and Flower, 2007a,b; Flower et al., 2010). More recently, Maritz-Olivier et al. (2012) used this tool for antigen prediction in the cattle tick, *R. microplus*, and identified 791 putative antigens, of which 176 were membrane-associated proteins including the Bm86 antigen. In a preliminary analysis of antigenicity, selected membrane proteins exhibited even better IgG-binding capacity than previously validated Bm86 epitopes, which were used as positive controls, pointing out that the potential of Vaxijen to predict protein antigens might also be extended to include ectoparasites. However, there is no successful (i.e., protective) vaccination experiments published to date using antigen candidates predicted by VaxiJen, so that VaxiJen could not be considered hitherto as a proven technique for predicting protective antigens for tick vaccines. Having these limitations in mind, we applied VaxiJen in the current study to identify putative antigenic proteins, which do not necessarily have to be protective, and to discard proteins that are not antigens.

The antigenic proteins distributed in the following categories: 1763 intracellular proteins, 131 secreted proteins and 516 membrane proteins.

Three hundred and ninety-six of the 516 predicted membrane proteins (76.7%) could be annotated and their sequences were inspected (Supplementary Table 1). Multi spanning proteins with more than four transmembrane domains, proteins known to be expressed on intracellular membranes and proteins with incomplete sequences were excluded from further analyses. The remaining 195 proteins were ranked according to their expression level, expression increase in fed ticks and predicted antigenicity. The top 30 proteins in each rank were

selected and combined giving a list of 75 unique proteins.

In order to select up to ten antigenic candidates among these 75 proteins and keep the subjectivity of the selection to a minimum, the proteins were manually revised and, in accordance with the hypothesis mentioned in the introduction section, priority was given to proteins predicted to be associated with the plasma membrane, to proteins that have previously been detected in the midgut proteome and to proteins annotated with functions that might be involved in processes related to blood digestion. In this way, six predicted proteins were selected as antigenic candidates to start their experimental production and testing in animal immunisation trials (Fig. 1, Table 1).

To this initial selection we added the Om86 protein, which we had identified previously (Oleaga et al., 2017b), despite the fact that its transcription level remained constant after feeding (fold change = 1) and, according to our selection criteria, it should have been excluded as a vaccine candidate.

However, the Om86 protein is the *O. moubata* orthologue of the Bm86 vaccine reference antigen (de la Fuente et al., 2007) and to the best of our knowledge no argasid orthologue of Bm86 has yet been tested in animal immunisation trials. In addition, the full-length sequence of the Om86 transcript/protein was verified and it largely met all the remaining requirements to be a potential vaccine candidate (Table 1), including the presence of the Om86 protein in the midgut proteome of fed ticks (Oleaga et al., 2017b). Therefore, we considered it essential to evaluate the vaccine efficacy of Om86 in animal immunisation trials, even although its molecular function and biological process could not be annotated and still are unknown.

The molecular function of the candidates Om85 and Om03 could not be annotated either and remain unknown. BLASTp analysis for Om85 reported 73% identity (100% coverage, E value 5×10^{-59}) with

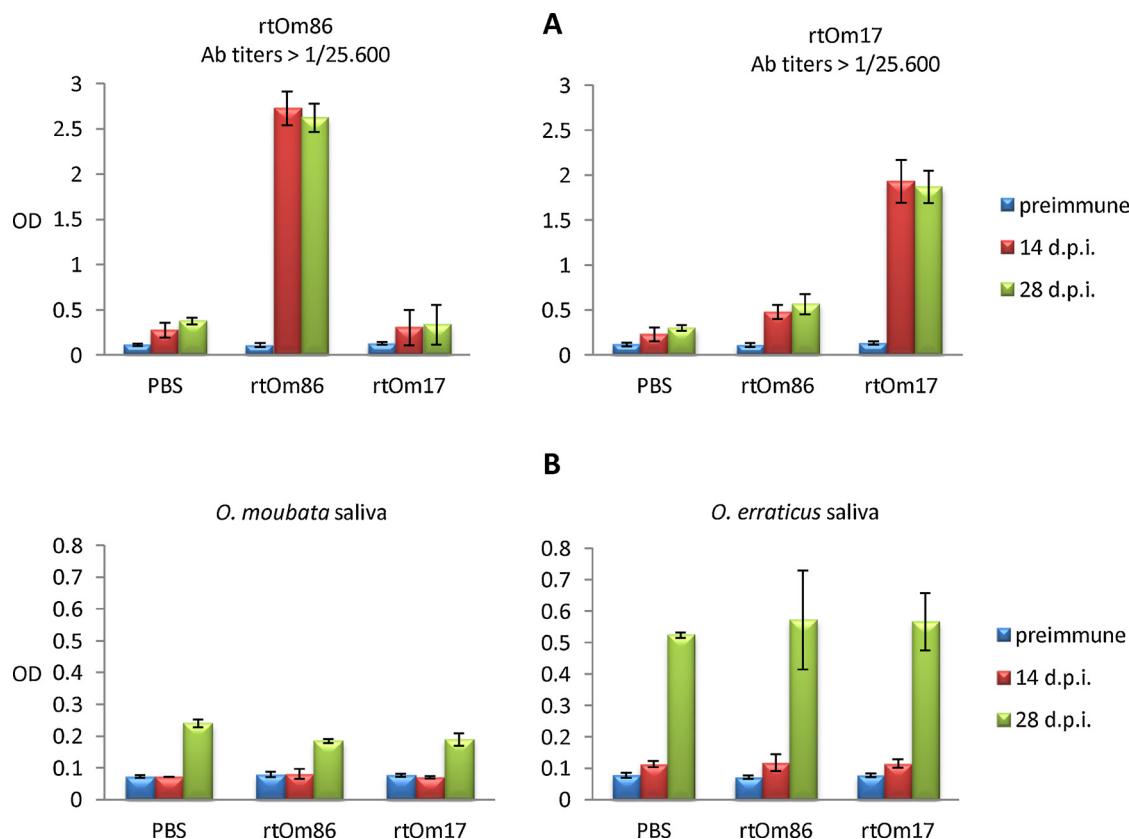


Fig. 4. ELISA. IgG antibody response in control rabbits (treated with PBS + Freund's) and in rabbits immunised with the rtOm86 and rtOm17 recombinant proteins. (A) Reactivity against each recombinant protein. (B) Reactivity against the saliva of *Ornithodoros moubata* and *O. erraticus*. Values are the average OD \pm SD at 492 nm for each rabbit group. Sera were taken before immunisation (preimmune) and at 14 and 28 days post-immunisation (d.p.i.), immediately before each infestation with ticks, and used at 1/300 dilution.

the transmembrane protein B7P5F1 of *Ixodes scapularis*, which belongs to the DUF2678 protein family (Pfam PF10856), of as yet unknown function. For Om03, BLASTp showed an 80% identity (98% coverage, E value 2×10^{-29}) with a conserved hypothetical protein of *Ixodes scapularis* (B7PMP9), which belongs to another functionally uncharacterised protein family UPF0542 (Pfam PF15086). Despite this, these proteins were considered potential candidates because of their high predicted antigenicity and increased transcription in fed ticks.

On the other hand, proteins Om99, Om29, Om28 and Om17 were annotated with molecular functions mainly related to transmembrane transport and surface signalling. In this context, these functions might be involved in cellular processes associated with nutrient transport and metabolism, which together with their high antigenicity (Om99, Om17) and/or strong upregulation in fed females (Om28, Om99) and/or high level of expression in fed female ticks (Om99, Om29), made these proteins interesting vaccine candidates (Sonenshine and Anderson, 2014; Oleaga et al., 2017a,b).

3.2. Predicted topologies of the candidate proteins

Table 2 and Fig. 2 show the predicted protein sequence and topology of the seven candidates. None of them have GPI anchors or non-classical secretion signals, and only Om86 has a signal peptide. Between one and four transmembrane domains were predicted for each candidate, which defined the following extracellular domains. Proteins Om86, Om29, Om28 and Om17 each have one large extracellular domain comprising 509, 90, 121 and 135 amino acids, respectively (Fig. 2A). These domains were selected for production as recombinant proteins.

On the other hand, proteins Om99, Om85 and Om03 each have only

one short amino-terminal extracellular domain comprising 24, 32 and 23 amino acids, respectively (Fig. 2B). As these domains are too small to be immunogenic (between 2700 and 3800 Da), they require conjugation to a carrier protein for use as antigens (Klein, 1990). Coupling to a carrier protein could be achieved by expressing the selected domains as recombinant fusion proteins. However, we found it easier and more convenient to design immunogenic peptides from their sequences – with the assistance of linear B-cell epitope prediction algorithms – and to synthesise and conjugate these peptides to a carrier protein.

3.3. Linear B-cell epitopes, design and synthesis of synthetic peptides

Fig. 3 shows the predicted linear B-cell epitopes on the extracellular domains of the Om99, Om85 and Om03 proteins. Three different immune-informatics tools predicted B-cell epitopes in all the candidates. The epitopes predicted by each tool on each of the candidates were somewhat different, but the sequences overlapped to a large degree, suggesting that the predictions were robust and reliable.

Based on these predictions, three peptides — one from each candidate — were designed (Table 3), synthesised and coupled to KLH as described in the materials and methods section 2.4.

3.4. Recombinant protein production

As indicated in the materials and methods section 2.5., expression of the recombinant rtOm29 and rtOm28 proteins failed and, although it was repeatedly attempted using different media (SOB, Psi), incubation temperatures (22 and 28 °C), higher IPTG concentrations (2 and 3 mM), longer induction times (5 and 6 h) and different host cells (*E. coli* SG13009, Qiagen), expression of these proteins was not achieved.

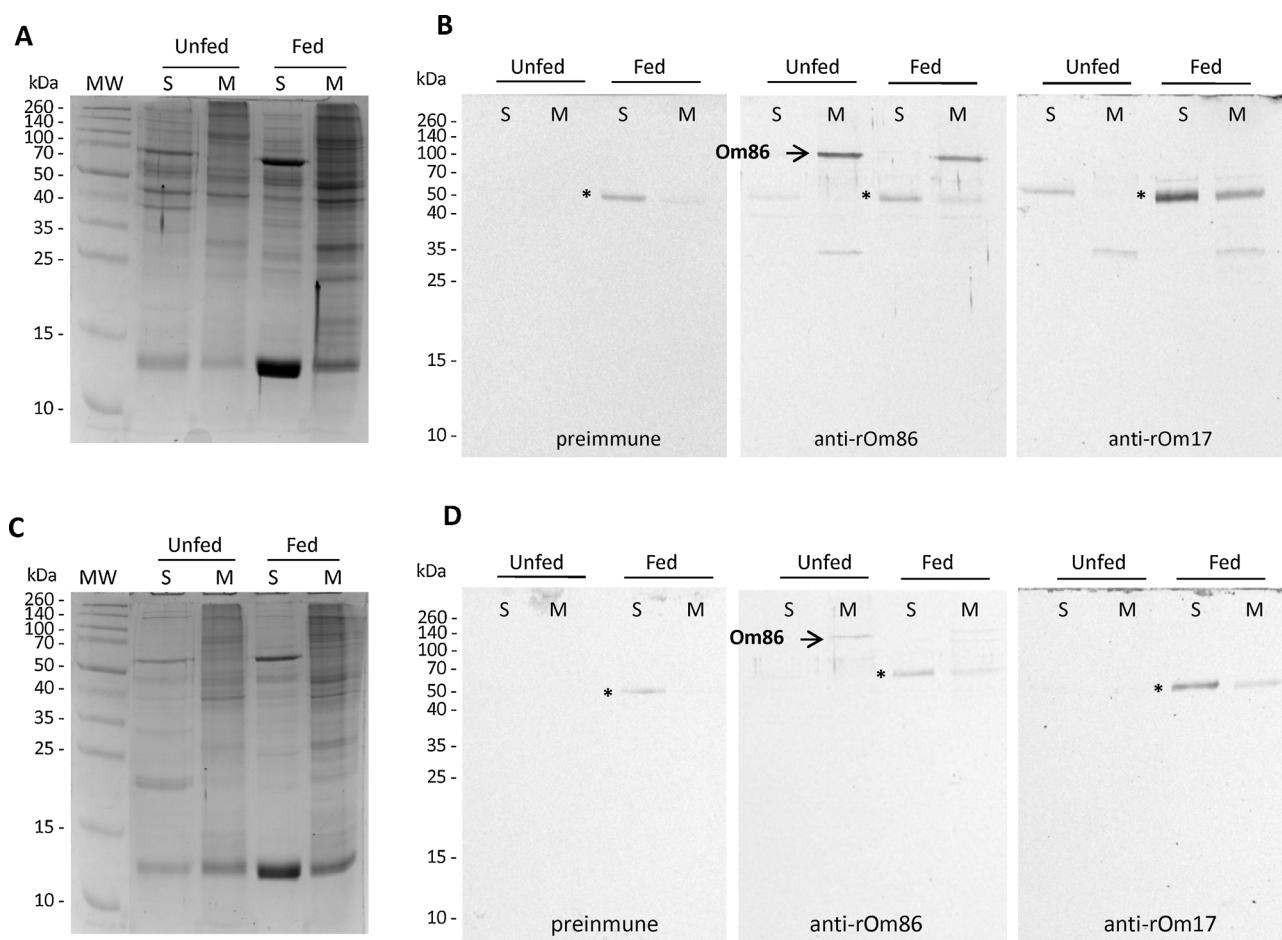


Fig. 5. (A, C) Coomassie Blue-stained 15% SDS-PAGE gels showing the soluble (S) and membrane (M) proteins of the midgut from *Ornithodoros moubata* (A) and *Ornithodoros erraticus* (C) female ticks taken before feeding (Unfed) and at 48 h after engorgement (Fed). (B, D) Western blots: antigens revealed by the sera from the rabbits immunised with the rtOm86 and rtOm17 recombinant proteins in the protein extracts from *O. moubata* (B) and *O. erraticus* (D). Sera were taken before immunisation (preimmune) and at 14 days post-immunisation (d.p.i.), immediately before the first infestation with ticks. Arrow: Om86 native protein in the membrane extracts. Asterisks: IgG heavy chain from the rabbit host, ingested with blood meal.

This failure was somewhat unexpected because we have routinely utilized prokaryotic expression systems for expression of recombinant proteins of ticks, in most cases with success (Díaz-Martín et al., 2015b). As an alternative, eukaryotic expression systems (yeast cells, insect cells or even plants) could have been a suitable way to express these two proteins. These systems are applied when the recombinant protein needs to be obtained as similar to the native protein as possible including its tertiary structure and post-transcriptional modifications (i.e., glycosylation) (Canales et al., 1997; Figlerowicz et al., 2013; Moreno-Cid et al., 2013). In fact, eukaryotic expression systems are more commonly used for the production of protective tick vaccines, for instance GAVAC® is produced in *Pichia pastoris* (Canales et al., 1997). Hence, we foresee use eukaryotic expression systems in coming studies.

The recombinant rtOm17 protein was abundantly expressed and easily purified to a single gel band showing the expected molecular weight of 16 kDa and a high yield of 23.6 mg per litre of culture (Supplementary Fig. 1A).

By contrast, the recombinant rtOm86 protein was expressed less abundantly and could not be solubilised either in 10% sarcosyl or 8 M urea, precluding its purification by nickel affinity chromatography. The presence of fifty cysteine residues in the expressed extracellular region (Table 2) suggested a very high probability of internal disulphide bond formation, which might account for its resistance to solubilisation. Actually, the addition of reducing agents such as 2-mercaptoethanol and DTT to the 8 M urea buffer resulted in the solubilisation of up to 30% of the recombinant rtOm86, lending support to the that idea.

Nonetheless, we considered this ratio of solubilisation insufficient to proceed with purification by nickel affinity chromatography, and we purified the insoluble rtOm86 directly from the cellular lysate pellet by electro-elution of the corresponding band from SDS-PAGE gels. The purified rtOm86 showed a molecular weight close to 75 kDa, markedly higher than the expected size of 57 kDa. This band was always accompanied in SDS-PAGE gels by two 35 kDa protein bands that could not be removed in any way, even after repeating the electroelution of the 75 kDa band. Analysis of these bands by LC-MS/MS confirmed the identity of the 75 kDa band as Om86 and that of the accompanying bands as chaperones from the *E. coli* host cells (Supplementary Fig. 1B). As a result, the electroeluted rtOm86 was recovered at 75.4% purity and yielded 164 µg per litre of culture (Supplementary Fig. 1B).

3.5. Vaccination trials. Humoral immune response and protective effects induced by the rtOm17 and rtOm86 antigens and the OM99, OM85 and OM03 peptides

A total of five candidates were produced, either as recombinant proteins (rtOm17 and rtOm86) or synthetic peptides (OM99, OM85 and OM03), and their individual protective potential was tested in animal immunisation trials. For convenience, these tests were carried out in two trials, testing the recombinant proteins and the synthetic peptides separately.

According to observations in previous studies, we supposed that the protective immune mechanisms induced by vaccination with these

Table 4

Effect of vaccination with rtOm86 and rtOm17 recombinant antigens on the *Ornithodoros moubata* and *Ornithodoros erraticus* specimens fed on control and vaccinated rabbits. Results are shown as mean \pm standard deviation for each rabbit group. Means were compared between ticks fed on vaccinated and control rabbits by one-way ANOVA followed by the Dunnett's t-test. Values inside parentheses represent the percentage of reduction in the corresponding parameter (or percentage increase for mortality rates) respect to the control.

Parameter	Life stage	First infestation			Second infestation		
		Control	rtOm86 (% reduction)	rtOm17 (% reduction)	Control	rtOm86 (% reduction)	rtOm17 (% reduction)
Ornithodoros moubata							
Ingested blood (mg)	Males	37.3 \pm 5.6	30.2 \pm 5.5 (18.9)	30.5 \pm 2.3 (18.2)	38.7 \pm 5.5	38.8 \pm 3.7	37.7 \pm 0.2 (2.5)
	Females	189.9 \pm 28.9	211.7 \pm 8.8	175.3 \pm 26.2 (7.7)	209.1 \pm 1.4	188.9 \pm 61.9 (9.7)	236.4 \pm 13.1
Mortality (%)	Nymphs-3	40.3 \pm 7.9	37.5 \pm 3.4 (7.1)	28.8 \pm 1.1 (28.6)*	16.7 \pm 2.1	16.3 \pm 0.8 (2.4)	14.9 \pm 7.4 (10.5)
	Males	0 \pm 0	4.1 \pm 3.6 (4.1)	2.7 \pm 4.0 (2.7)	1.3 \pm 2.2	0 \pm 0	1.3 \pm 1.9
	Females	0 \pm 0	6.7 \pm 5.9 (6.7)	2.2 \pm 3.3 (2.7)	6.6 \pm 11.5	8.3 \pm 6.5 (1.7)	0 \pm 0
Oviposition (eggs/female)	Nymphs-3	3.3 \pm 1.1	3.7 \pm 1.1 (0.4)	4.7 \pm 3.7 (1.4)	2.7 \pm 2.4	0.7 \pm 1.0	4.1 \pm 3.2 (1.4)
Fertility (nymphs/female)	Females	173.8 \pm 20.1	180.6 \pm 15.9	151.5 \pm 36.2 (12.8)	195.1 \pm 3.1	205.4 \pm 62	276.2 \pm 25.4
	Females	153.6 \pm 23.4	152.2 \pm 15.5	129.9 \pm 23.8 (15.4)	189.2 \pm 4.5	195.3 \pm 56.7	265.2 \pm 19.7
Ornithodoros erraticus							
Ingested blood (mg)	Males	3.9 \pm 0.1	3.9 \pm 0.2	3.8 \pm 0.1 (2.5)	4.1 \pm 1.2	4.9 \pm 2.2	5.6 \pm 1.1
	Females	10.5 \pm 3.0	13.5 \pm 2.9	14.7 \pm 1.5	10.5 \pm 0.6	15.9 \pm 1.5	11.9 \pm 1.3
Mortality (%)	Nymphs-3	2.1 \pm 0.2	2.3 \pm 0.3	2.6 \pm 0.1	2.5 \pm 0.9	1.1 \pm 1.5	2.1 \pm 0.5
	Males	2.8 \pm 2.4	1.3 \pm 2.8	2.7 \pm 4.0	1.3 \pm 2.3	0 \pm 0	1.4 \pm 2.1 (7.7)
	Females	0 \pm 0	0 \pm 0	4.8 \pm 4.1 (4.8)*	0 \pm 0	0 \pm 0	0 \pm 0
Oviposition (eggs/female)	Nymphs-3	3.2 \pm 3.9	0.7 \pm 1.1	2.8 \pm 31.1	2.3 \pm 4.0	0 \pm 0	2.2 \pm 2.0
Fertility (larvae/female)	Females	36.3 \pm 2.6	49.0 \pm 18.0	53.5 \pm 7.2	49.9 \pm 13.6	64.2 \pm 16	51.4 \pm 8.2
	Females	7.1 \pm 0.1	7.6 \pm 4.1	4.6 \pm 5.3 (35.2)	44.2 \pm 12	55 \pm 2.9	43.1 \pm 4.2 (2.5)

* $p < 0.05$.

** $p < 0.01$.

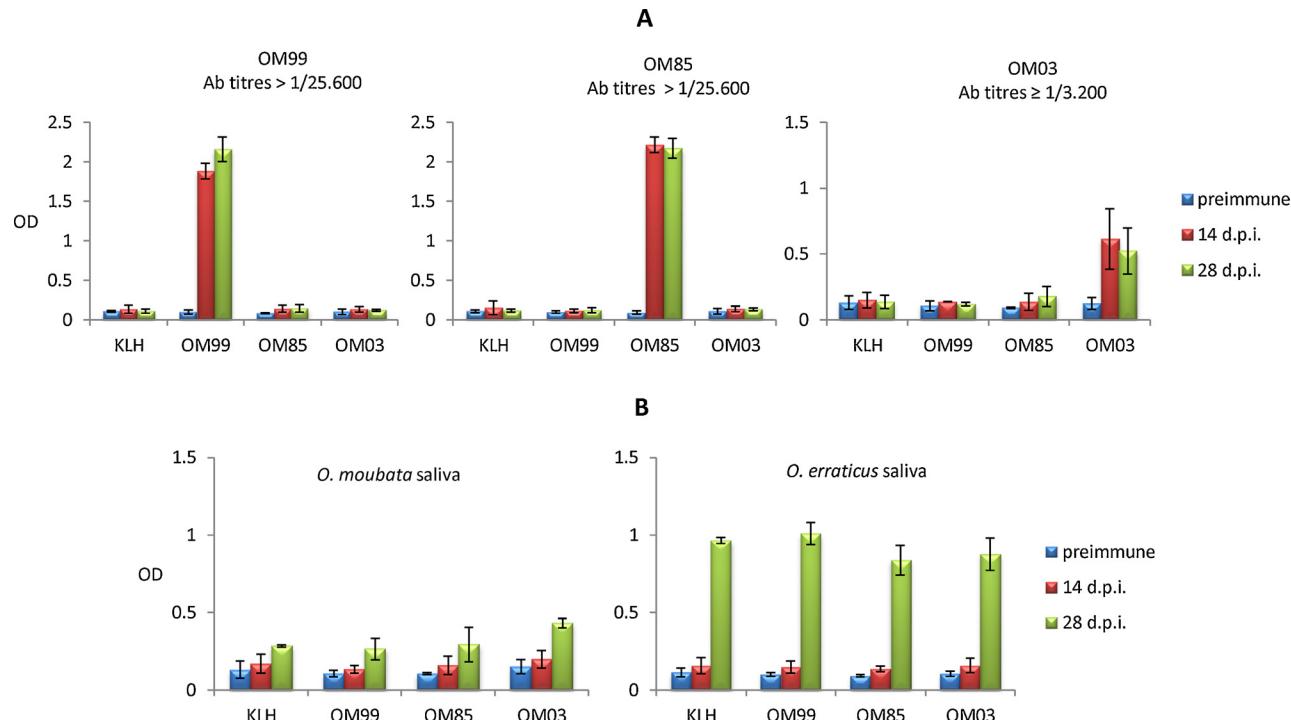


Fig. 6. ELISA. IgG antibody response in control rabbits (treated with KLH) and rabbits immunised with the KLH-conjugated peptides OM99, OM85 and OM03. (A) Reactivity against each synthetic peptide. (B) Reactivity against the saliva of *Ornithodoros moubata* and *Ornithodoros erraticus*. Values are the average OD \pm SD at 492 nm from each rabbit group. Sera were taken before immunisation (preimmune) and at 14 and 28 days post-immunisation (d.p.i.), immediately before each infestation with ticks, and used at 1/300 dilution.

candidates will likely be mediated by antibodies, which would bind to their target antigen and neutralize its biological function or interact with the complement system – or both- disrupting the enterocyte function and integrity. We also assumed that protection will be directly related to the level of specific antibodies and to antibody quality, i.e., avidity and affinity (Manzano-Román et al., 2006, 2007; Díaz-Martín et al., 2015a,b; Maruyama et al., 2017; Andreotti et al., 2018).

The sera from rabbits immunised with rtOm86 and rtOm17 recognised their corresponding recombinant proteins with high specificity and reactivity (Supplementary Fig. 1C; Fig. 4A). It is worth mentioning that the sera taken at 14 d.p.i., before the first infestation, did not react to the saliva of *O. moubata* or *O. erraticus* (Fig. 4B), indicating that these two midgut proteins do not share epitopes with salivary proteins. Not surprisingly then, tick bites in the immunised hosts did

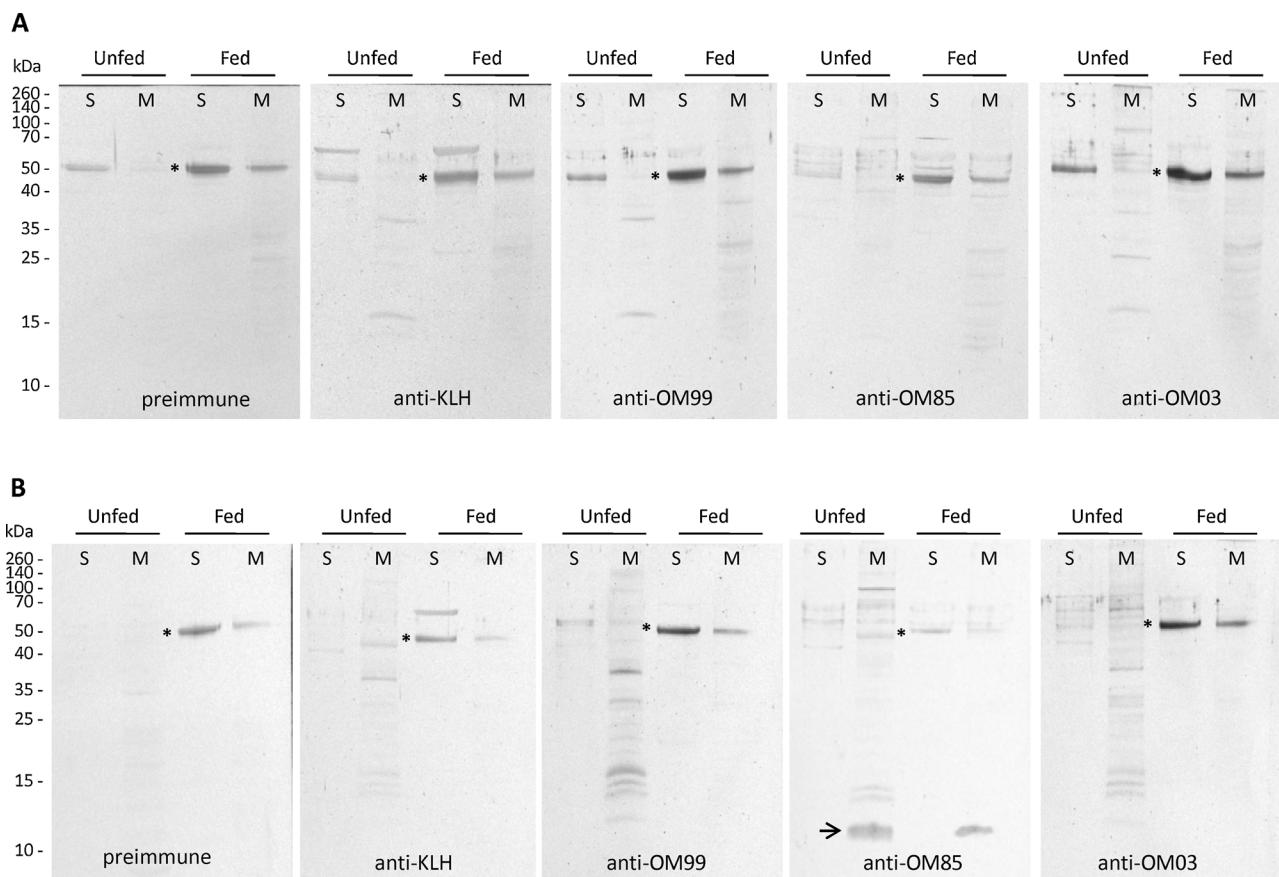


Fig. 7. Western blot: antigens revealed by the sera from the rabbits immunised with KLH and the KLH-conjugated peptides OM99, OM85 and OM03 among the soluble (S) and membrane (M) proteins of the midgut from *Ornithodoros moubata* (A) and from *O. erraticus* (B) female ticks taken before feeding (Unfed) and at 48 h after engorgement (Fed). These protein extracts are the same as those shown in Fig. 5 (A, C). Sera were taken before immunisation (preimmune) and at 14 days post-immunisation (d.p.i.), immediately before the first infestation with ticks. Arrow: Om85 native protein in the membrane extracts. Asterisks: IgG heavy chain from the rabbit host, ingested with blood meal.

not enhance the vaccine-induced immune response, as the slight decrease in the reactivity of the sera taken at 28 d.p.i. indicates (Fig. 4A). Thus, natural tick-host contacts in the vaccinated hosts would not serve to boost antigen doses.

Regarding the native Om86 and Om17 proteins from the midgut extracts (Fig. 5), it was observed that the anti-rtOm86 serum pool recognised the native Om86 protein (as a band of 85 kDa) from extracts of membrane proteins, but not from soluble proteins, from fed and unfed *O. moubata* females (Fig. 5B; Supplementary Fig. 2A). This further supports the predicted localisation of Om86 at the enterocyte plasma membrane. The anti-rtOm86 serum pool also recognised, although more weakly, a similar band of 85 kDa among the membrane proteins from *O. erraticus* females (Fig. 5D; Supplementary Fig. 2B), indicating that the Om86 protein and its native orthologue in *O. erraticus* (the so called Oe86) share cross-reactive epitopes with similar localisation in the enterocyte.

On the other hand, the anti-rtOm17 serum pool did not recognise any band with the characteristics of the native Om17 protein from any midgut extract from *O. moubata* or *O. erraticus* (Fig. 5B, D). This lack of recognition and its absence in the *O. moubata* midgut proteome (Oleaga et al., 2017b) strongly suggest that Om17 is poorly expressed in the midgut at the two time-points sampled during the tick trophogenic cycle (unfed, 48 hp.f.). As gene transcription and translation have different regulation mechanisms, transcriptome and proteome kinetics can be quite different (Maier et al., 2009; Kumar et al., 2016). Thus, although the Om17 mRNA level increased in fed females at 48 hp.f. (Table 1), expression of the Om17 protein might take place at a different point of the trophogenic cycle, not sampled in the current study.

The protective effect of the immune response induced by the rtOm17 and rtOm86 recombinant proteins was low in the first infestation and negligible in the second (Table 4). Protection against *O. moubata* essentially consisted in: (i) reductions in feeding performance, which were significant only in nymphs-3 fed on rabbits immunised with rtOm17; (ii) slight increases in mortality at all tick life stages; and (iii) reduced oviposition in females fed on rabbits immunised with rtOm17. Thus, the vaccine efficacy of rtOm86 and rtOm17 against *O. moubata* was 7% and 18.5%, respectively, for the first infestation, and below 1% for the second infestation (Supplementary Table 4, Supplementary Fig. 4). Regarding *O. erraticus*, the immune response to rtOm86 did not affect any parameter in any infestation, while the response to rOm17 only affected the females in the first infestation by reducing their survival and fertility. Thus, rtOm86 was not effective, and rtOm17 showed a 36.9% efficacy in the first infestation which vanished in the second infestation.

The results of this vaccine trial indicated that the rtOm86 antigen was only slightly effective against *O. moubata* and ineffective against *O. erraticus*, although the sera of the vaccinated animals recognised the native Om86 and its *O. erraticus* orthologue (Oe86) in extracts of midgut membrane proteins.

By contrast, the rtOm17 antigen was effective against both species, although the native Om17 protein was not recognised by the immune sera in the midgut protein extracts from unfed and 48 hp.f. fed females. The protection provided by rtOm17 strongly suggests that native Om17 protein must be expressed at some time point along the trophogenic cycle and its function is somehow neutralized by the vaccine-induced antibodies. Although the current results do not allow knowing when the

Table 5
Effect of vaccination with the KLH-conjugated peptides OM99, OM85 and OM03 on the *Ornithodoros moubata* and *Ornithodoros erraticus* specimens fed on control (treated with KLH) and vaccinated rabbits. Results are shown as mean \pm standard deviation for each rabbit group. Means were compared between ticks fed on vaccinated and control rabbits by one-way ANOVA followed by the Dunnett's t-test. Values inside parentheses represent the percentage of reduction in the corresponding parameter (or percentage increase for mortality rates) respect to the control.

Parameter	Life stage	First infestation		Second infestation		OM03 (% reduction)	OM85 (% reduction)	OM99 (% reduction)	Control KLH
		Control KLH	OM99 (% reduction)	Control KLH	OM99 (% reduction)				
Ornithodoros moubata									
Ingested blood (mg)	Males	27.6 \pm 3.1	33.5 \pm 4.6	22.5 \pm 3.5 (18.5)*	35.5 \pm 0.7	40.8 \pm 2.3	32.3 \pm 2.8 (18.1)*	29.0 \pm 3.9 (26.3)*	37.3 \pm 4.1 (5.2)*
	Females	199.3 \pm 15.7	211.6 \pm 16.5	192.2 \pm 30.1 (3.6)	198.2 \pm 5.4 (0.6)	192.5 \pm 7.2 (7.6)	192.5 \pm 7.2 (7.6)	169.1 \pm 18.8 (18.8)*	185.1 \pm 8.2 (11.2)*
Nymphs-3	Males	8.8 \pm 0.7	8.6 \pm 0.9 (2.2)	7.9 \pm 1.2 (10.2)	8.5 \pm 1.3 (3.4)	8.6 \pm 0.7	8.0 \pm 0.4 (6.9)*	9.5 \pm 0.5	11.2 \pm 0.5
	Females	1.3 \pm 1.9	4.5 \pm 0.1 (3.2)*	3.3 \pm 4.9 (2.0)	2.7 \pm 1.9 (1.4)	4.0 \pm 5.9	2.7 \pm 1.9	1.3 \pm 1.9	0 \pm 0
Mortality (%)	0 \pm 0	0 \pm 0	0 \pm 0	4.4 \pm 3.29 (4.4)*	2.2 \pm 3.3	4.6 \pm 3.4 (2.4)	0 \pm 0	4.4 \pm 6.4 (2.2)	
Oviposition (eggs/female)	Nymphs-3	0.7 \pm 1.0	0 \pm 0	0 \pm 0	1.4 \pm 2.1 (0.7)	0 \pm 0	2.1 \pm 1.7 (2.1)*	0.7 \pm 1.0 (0.7)	0 \pm 0
Fertility (nymphs/female)	Females	229.8 \pm 5.6	213.7 \pm 6.8 (7.1)*	158.1 \pm 1.8 (31.2)*	193.4 \pm 16.4 (15.8)*	220.1 \pm 22.8	190.9 \pm 16.7 (13.2)*	207.5 \pm 18.1 (5.6)	187.0 \pm 15.6 (15)*
Ornithodoros erraticus	Females	219.5 \pm 3.7	201.4 \pm 10.7 (8.1)*	136.7 \pm 25.5 (37.4)*	185.1 \pm 18.1 (15.4)*	209 \pm 17.5	178.8 \pm 14.2 (14.4)*	197.2 \pm 18.4 (5.7)	173.9 \pm 20.9 (16.7)*
Ingested blood (mg)	Males	3.4 \pm 0.2	3.9 \pm 0.2	3.2 \pm 0.1 (5.9)	4.5 \pm 1.2	4.1 \pm 0.2	3.9 \pm 0.1 (4.9)	3.0 \pm 0.4 (26.8)*	3.9 \pm 0.1 (0.2)
	Females	14.7 \pm 4.1	13.4 \pm 2.8 (8.8)	11.8 \pm 1.5 (19.7)*	12.1 \pm 1.8 (17.7)*	12.5 \pm 0.6	11.8 \pm 0.9 (5.6)	9.7 \pm 1.2 (22.4)*	10.8 \pm 1.6 (13.6)*
Nymphs-3	Males	2.3 \pm 0.1	2.6 \pm 0.5	2.9 \pm 0.2	2.8 \pm 0.9	2.8 \pm 0.2	2.4 \pm 0.3 (14.3)	2.4 \pm 0.28 (14.3)	2.5 \pm 0.3 (10.7)
	Females	2.7 \pm 2	0 \pm 0	2.8 \pm 2 (0.1)	1.3 \pm 1.9	1.4 \pm 2.1	0 \pm 0	1.3 \pm 1.9	1.4 \pm 2.0
Mortality (%)	0 \pm 0	0 \pm 0	2.1 \pm 0.3 (2.1)*	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	2.2 \pm 3.2 (2.2)*
Oviposition (eggs/female)	Nymphs-3	2.4 \pm 1	3.9 \pm 1.6 (1.5)	13.4 \pm 35.6 (1.1)*	2.5 \pm 1.9 (0.1)	2.1 \pm 0.2	2.2 \pm 3.2 (0.1)	7.0 \pm 1.0 (4.9)*	7.1 \pm 4.7 (5)*
Fertility (larvae/female)	Females	40.3 \pm 5.6	34.6 \pm 9.7 (14.1)	18.3 \pm 9.6 (54.6)*	18.4 \pm 7.0 (54.3)*	36.2 \pm 3.5	25.8 \pm 5.6 (28.7)*	11.8 \pm 2.8 (67.4)*	19.4 \pm 5.7 (46.4)*
	Females	36.5 \pm 4.3	28.9 \pm 6.7 (20.1)*	14.7 \pm 8.5 (59.7)*	12.4 \pm 6.6 (66.1)*	19.2 \pm 11.8	9.9 \pm 13 (48.4)*	6.4 \pm 2 (66.7)*	8.8 \pm 5.6 (54.2)*

* $p < 0.05$.** $p < 0.01$.

Om17 protein expression took place, it is reasonable to think that such expression will have occurred after 48 h.p.f., following the 2.05 fold-increase in the Om17 mRNA level. Measurements of Om17 mRNA levels and/or quantification of native Om17 protein in midgut samples taken at different points along the trophogenic cycle might have helped explain these results more objectively; however, none of these assays have been performed in this work.

The protection provided by rtOm17 was observed only in the first infestation, which can be easily explained, but the level of protection against *O. erraticus* was greater than against the homologous species, *O. moubata*, which has a less obvious explanation, as we will discuss below.

The lack of protection of Om86 against *O. erraticus* is not surprising considering that the Bm86-based vaccines are effective against closely related species such as *R. annulatus* or *R. decoloratus*, but not against more distantly related species (Willadsen, 2008; Canales et al., 2009; de la Fuente and Kocan, 2014). Since *O. erraticus* is a relatively distant species from *O. moubata* (Vial, 2009; Trape et al., 2013), it can be supposed that the Om86 and its *O. erraticus* orthologue (Oe86) do not share enough sequence identity to ensure that specific antibodies raised against Om86 bind to Oe86 with the necessary affinity to trigger and maintain the immune effector mechanism responsible for the protection, i.e., the activation of the complement system on the luminal membrane of the enterocyte damaging the midgut wall (Willadsen, 2008).

The low efficacy of rtOm86 against the homologous species, *O. moubata*, is more challenging to explain. It could be the result of several of factors such as: (i) the existence of polymorphisms in the sequence of Om86 (Oleaga et al., 2017a); (ii) a low level of protein expression; (iii) an insufficient amount of ingested blood and thus insufficient amount of specific antibodies; or (iv) the presence of abundant proteases in the midgut after feeding (Oleaga et al., 2017b) that could degrade the antibodies. These same factors have also been proposed by other authors to explain differences in protective efficacy of the Bm86 antigen among different strains of *R. microplus* (Popara et al., 2013; de la Fuente and Kocan, 2014; Coumou et al., 2015).

Additionally, the formation of a peritrophic matrix (PM) in the tick midgut lumen could have impacted the vaccine outcome. The PM separates ingested blood from the intestinal epithelium protecting the enterocytes against particulate or infectious elements (Lehane, 1997). The PM might hinder the access of the antibodies and the complement to their targets on the surface of the enterocytes, reducing the vaccine efficacy. The presence of PM has been documented in several species of ixodids (Sojka et al., 2013) and was described some time ago in *O. moubata* by Grandjean (1983). More recently, Oleaga et al. (2017a) found that 12 of the 19 genes potentially involved in the formation of the PM were upregulated in the tick midgut at 48 h.p.f. suggesting the presence of this structure in fed females. By contrast, no evidence of PM formation has yet been found in the midgut proteome of fed *O. erraticus* females (Oleaga et al., 2015). Therefore, it could be speculated that the lack of a PM — or the formation of a thinner and more permeable PM — in *O. erraticus* could contribute to the higher efficacy of the anti-rtOm17 response in this species compared with *O. moubata*.

Recent studies have demonstrated that diverse bacteria and fungi inhabit the tick gut and that these microorganisms are essential to tick physiology and the interaction between ticks and tick-borne pathogens (Narasimhan et al., 2014; Abraham et al., 2017). The tick gut microbiota have been demonstrated to modulate the expression of tick genes such as the transcription factor STAT, which in turn regulates the expression of genes involved in tick immune responses and epithelial repair and remodelling. One such gene is peritrophin, a scaffold glycoprotein that maintains the structural integrity of the PM (Hegedus et al., 2009; Narasimhan et al., 2014). Thus, it could be that a different gut microbial composition might have a different impact on the vaccine outcome, either through modulation of PM formation or by some other as yet unknown mechanism. Although plausible, this idea should be

experimentally tested.

As previously stated, in the current work we have worked in the assumption that the protective mechanism of these vaccines is mediated by specific antibodies. Thus, according to Willadsen (2008), the efficacy of the vaccine will depend on achieving and maintaining a strong humoral response. As our antigens Om86 and Om17 do not share epitopes with salivary proteins (Fig. 4), the natural infestations did not act as boosting antigen doses and the level of specific antibodies decreased by the time of second infestation. The decrease was slight, but may be enough to reduce the antibody level below the efficacy threshold, which would explain the lack of protection with either antigen in the second infestation.

The synthetic peptides analysed in the second vaccine trial induced strong (anti-OM99, anti-OM85) and intermediate (anti-OM03) humoral responses, demonstrating high immunogenicity in accordance with the linear B-cell epitope predictions (Fig. 3). These responses specifically recognised the inducing peptide (Fig. 6A) and did not react to the saliva of *O. moubata* or *O. erraticus* (Fig. 6B), showing that the synthetic peptides do not share cross-reactive epitopes with each other or with salivary proteins. Accordingly, the tick bites did not boost the vaccine-induced immune response indicating that natural tick-host contacts would not serve as enhancing antigen doses in immunised hosts (Fig. 6A).

These immune sera poorly reacted to soluble and membrane midgut proteins of both *Ornithodoros* species according to ELISAs (Supplementary Fig. 3) and most of the reactivity was due to the non-specific recognition of host IgG and other proteins which were also recognised by the preimmune and anti-KLH sera (Fig. 7). None of the immune sera detected any specific band in any of the *O. moubata* extracts (Fig. 7A), and only the anti-OM85 sera revealed one specific band of 12 kDa among the membrane proteins of *O. erraticus*, which was compatible in size with the native Om85 protein, showing cross-reactive epitopes with the *O. erraticus* orthologue of Om85 (Os85) (Fig. 7B).

As suggested for the Om17 protein in the first trial, the lack of recognition of the native Om99 and Om03 proteins — and of native Om85 in *O. moubata* — could be explained if their expression occurred transiently at a time point during trophogenic cycle different from those currently sampled (unfed and 48 h.p.f.).

The effect of the immune response induced by the peptides on the *O. moubata* ticks was moderate-low in the first infestation and low in the second (Table 5). The three peptides caused small reductions in the amount of blood ingested, and moderate reductions in the oviposition and fertility of the females, hardly affecting the survival of the specimens. Accordingly, in the first infestation, the three peptides showed protective efficacies between 38.6% and 12.8%, with peptide OM85 being the most effective, followed by OM03 and OM99. In the second infestation, the efficacy of OM85 decreased dramatically to 2.7%, while the efficacies of OM03 and OM99 declined only moderately, remaining at a similar level as in the first infestation (Supplementary Table 4, Supplementary Fig. 4).

The effect of the immune response on *O. erraticus* was qualitatively similar, but more intense, compared with that observed in *O. moubata*. The three peptides caused significant reductions in the amount of blood ingested by the females, which resulted in consistent reductions in their fertility. The peptides also caused some reductions in the survival of the ticks, although not always significant (Table 5). Thus, in the first infestation, all of the peptides showed significant protective efficacies (from 20.7% to 66.1%), with OM03 being the most effective, followed by OM85 and OM99. In the second infestation, the efficacy of OM85 remained practically unchanged, becoming the most effective (55.1%), while the efficacy of OM03 decreased to 38.5% and that of OM99 increased to 30% (Supplementary Table 4, Supplementary Fig. 4).

The fact that all three peptides provided protection against *O. moubata* and particularly against *O. erraticus* raises some interesting points that deserve further comment. First, despite their lack of

reactivity in ELISAs and western blots (except for Om85), these immune sera actually interacted with their targets on the midgut membrane, supporting the idea that these proteins are likely expressed at times of the trophogenic cycle other than those analysed here. Second, the Om99, Om85 and Om03 proteins share protective epitopes with their orthologues in *O. erraticus*, which enhances their value as broader spectrum vaccine antigens. Third, the fact that they are more effective against *O. erraticus* than *O. moubata* suggests that there may be particular factors in *O. moubata* (anatomical, physiological, molecular or microbial) that reduce the efficacy of the midgut concealed antigens against this species. This higher sensitivity of *O. erraticus* to midgut antigens compared with *O. moubata* had already been observed by our team in previous works in which rabbit hosts were vaccinated with crude extracts of midgut membranes from each of these species (García-Varas, 2004; Manzano-Román et al., 2006, 2007). Finally, it should also be noted that since the peptides do not share epitopes with saliva proteins, tick bites will not boost the vaccine-induced immune response and, consequently, it is expected that the level of specific antibodies and protective efficacy will decrease with time, as was observed in the second infestation (Table 5).

In summary, the synthetic peptides were more efficient than the recombinant proteins, more effective against *O. erraticus* than against *O. moubata*, and more effective in the first infestation compared with the second. Regarding the recombinant proteins, rtOm86 was poorly effective only against *O. moubata* in the first infestation, whereas rtOm17 was somewhat effective, but only in the first infestation, and was more effective against *O. erraticus* than against *O. moubata*.

4. Conclusions

The recent availability of transcriptomic and proteomic data from the *O. moubata* midgut has allowed us to implement a bioinformatics-based strategy in which we used criteria based on hypotheses of predicted antigenicity, cellular localisation, functionality and over-expression after feeding to select potentially protective concealed antigens from the tick midgut.

The evaluation of five theoretical candidates in animal vaccine trials demonstrated low or very low protective potential for candidates Om17, Om86 and Om99, and significant moderate protection for candidates Om85 and Om03. These results suggest that the candidates Om17, Om86 and Om99 are of little value for their use as antigens in vaccines against *Ornithodoros* ticks, whereas peptides OM85 and OM03 might be candidates to be integrated in the development of soft tick vaccines and they deserve further studies.

None of the candidates share epitopes with salivary proteins of *O. moubata* or *O. erraticus*, so the natural contacts with these species do not act to boost antigen doses.

All candidates, except for Om86, share protective epitopes in *O. moubata* and *O. erraticus*. Notably, these candidates provided greater protection against *O. erraticus* than against *O. moubata*, confirming previous observations about the lower sensitivity of *O. moubata* to vaccines based on concealed antigens from the midgut. This lower sensitivity could be attributed to anatomo-physiological peculiarities of *O. moubata*, which may not be present in *O. erraticus*.

As a whole, these observations suggest that the midgut and the mialoma might not be the best choice as a source of candidate antigens for anti-*O. moubata* vaccines, but they do not rule out midgut antigens as candidates for anti-*O. erraticus* vaccines.

It should be also considered if the pipeline for candidate selection used in this work was the best option. VaxiJen has not hitherto been proven as a tool for predicting protective antigens from ectoparasites, and the current results suggest that VaxiJen would not be a useful predictor for *Ornithodoros* tick protective antigens.

Thus, alternative methods to identify *O. moubata* vaccine candidates would have to focus on other organs and systems (salivary glands, reproductive organs, etc.) and refine selection criteria. New candidate

protective antigens will likely be identified by focusing on proteins with relevant biological functions in key physiological processes for tick survival and tick-host-pathogen interactions. In this regard, it seems that global system biology analysis of the host-tick-pathogen interactions at these other organs and systems could be the best option to acquire abundant “omic” data whose integration and analysis will facilitate the identification of key tick proteins and functions and their selection as vaccine candidates.

Funding

This work was supported by project AGL2013–42745-P funded by a grant from the Spanish Ministry of Economy and Competitiveness.

Acknowledgements

The authors are grateful to Rocío Vizcaíno-Marín, María González-Sánchez and Diana Ramos-Pulido from the Instituto de Recursos Naturales y Agrobiología de Salamanca (IRNASA, CSIC) (Spain), for their skilful technical assistance.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.ttbdis.2018.04.015>.

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