Title

New salivary anti-haemostatics containing protective epitopes from *Ornithodoros moubata* ticks: assessment of their individual and combined vaccine efficacy

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Abstract

Ornithodoros moubata is the main vector of the pathogens causing African swine fever and human relapsing fever in Africa. The development of an efficient vaccine against this tick would facilitate its control and the prevention of the diseases it transmits to a considerable extent. Previous efforts to identify vaccine target candidates led us to the discovery of novel salivary proteins that probably act as anti-haemostatics at the host-tick interface, including a secreted phospholipase A_2 (PLA2), a 7DB-like protein (7DB-like), a riboprotein 60S L10 (RP-60S), an apyrase (APY), and a new platelet aggregation inhibitor peptide, designated mougrin (MOU). In this work, the corresponding recombinant proteins were expressed in *Escherichia coli* and their individual vaccine efficacy was tested in rabbit vaccination trials. All of them, except the less immunogenic RP-60S, induced strong humoral responses that reduced tick feeding and survival, providing vaccine efficacies of 44.2%, 43.2% and 27.2%, 19.9% and 17.3% for PLA2, APY, MOU, RP-60S and 7DB-like, respectively. In the case of the more protective recombinant antigens (PLA2, APY and MOU), the immunodominant protective linear B-cell epitopes were identified and their combined vaccine efficacy was tested in a second vaccine trial using different adjuvants. In comparison with the best efficacy of individual antigens, the multicomponent vaccine increased vaccine efficacy by 13.6%, indicating additive protective effects rather than a synergistic effect. Tick saliva inoculated during natural tick-host contacts had a boosting effect on vaccinated animals, increasing specific antibody levels and protection.

Keywords

Ornithodoros moubata; salivary antigens; vaccine; recombinant antigens; B-cell epitopes.

Highlights

- Five new recombinant salivary anti-haemostatics from *O. moubata* were tested as vaccine antigens.
- Recombinant PLA2, APY and MOU induced valuable protective responses.
- Joint administration of these three protective antigens increased vaccine efficacy.
- Tick infestations boost protection by acting as natural re-vaccination events.

1. Introduction.

The argasid tick *Ornithodoros moubata* is distributed throughout South and East Africa and Madagascar, where it colonizes wild and domestic habitats and feeds on warthogs, domestic swine and humans (Vial, 2009). *O. moubata* transmits both the African swine fever (ASF) virus and the tick-borne human relapsing fever (TBRF) agent *Borrelia duttoni*. The presence of this tick in domestic and peridomestic 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). Therefore, the elimination of *O. moubata* from synanthropic environments would greatly improve the prevention and control of such diseases.

Anti-tick vaccines have proved to be a feasible, cost-effective and environmental-friendly method for the control of tick infestations, avoiding many of the drawbacks associated with the use of chemical acaricides (de la Fuente et al., 2007; Willadsen, 2008a; Guerrero et al., 2012).

Investigations for the development of vaccines against *O. moubata* began with Chinzei and Minoura (1988) and Astigarraga et al. (1995), who tested the protective effect of concealed antigens from tick eggs and exposed antigens from tick salivary glands, respectively. Subsequent investigations using salivary and concealed antigens have identified some promising vaccine candidates, but currently an effective vaccine against *O. moubata* is still lacking (Díaz-Martín et al., 2015).

Regarding studies involving salivary antigens, Astigarraga et al. (1995) observed that pig vaccination with salivary gland extracts (SGE) induced protective responses, and that the sera from protected pigs recognized an antigen of 44 kDa (hence the name Om44), which was never recognized by the sera from non-protected pigs sensitized by natural infestation. In addition, vaccination with the purified Om44 antigen was shown to provide similar protection to SGE and this protection was observed to increase with successive *O. moubata* infestations (García-Varas et al., 2010). The functional characterization of Om44 revealed that it acts as an antagonist ligand for host P-selectin, preventing the P-selectin-mediated haemostatic and

inflammatory host response at the tick bite site and hence enabling ticks to complete their blood feeding (Cleator et al., 2006; García-Varas et al., 2010).

The identification of Om44 was first attempted by mass spectrometry; this was unsuccessful, although the mass spectrometric data did reveal that Om44 was not an orthologue of well-established P-selectin ligands, including PSGL-1 and pentraxin 3 (García-Varas et al., 2010). Next, taking into account that protein microarrays are powerful tools that allow the identification of molecular partners interacting at the host-pathogen interface (Manzano-Román et al., 2013), self-assembled nucleic acid programmable protein microarrays (NAPPA) were constructed from *O. moubata* salivary gland cDNA expression libraries. In order to identify Om44, these arrays were screened using a P-selectin/IgG chimera and an anti-Om44 polyclonal serum as probes. The results of this screening indicated that Om44 is a salivary secreted phospholipase A_2 (PLA2) that binds P-selectin through protein-protein interactions without the involvement of glycan moieties (Manzano-Román et al., 2012). Consequently, it was speculated that this PLA_2 might be an important candidate for vaccine development. Additionally, the anti-Om44 serum recognized another four salivary proteins in the microarray screening: a putative 7DB family member (7DB-like), riboprotein 60S L10 (RP-60S), heat shock protein 90 (HSP90) and actin (Manzano-Román et al., 2012). With the exception of actin, these proteins might have also contributed to the protective responses induced by the SGE and the purified Om44 antigen, so they could also be regarded as candidate antigens for vaccine development. Actin was excluded as a potential vaccine antigen owing to its high degree of conservation between ticks and mammals and to the lack of protective effects observed by us in a preliminary vaccine trial (unpublished) and by other authors using actin in vaccine trials against other parasites (Zheng et al., 2013).

Moreover, a preliminary partial random sequencing of the above-mentioned libraries allowed us to identify two novel salivary proteins of *O. moubata*: an apyrase (APY) and a platelet-aggregation inhibitor peptide orthologous to the savignyigrin of *O. savignyi*, which was called mougrin (MOU) (Manzano-Román et al., 2012; Díaz-Martín, 2014). Owing to the potential anti-haemostatic activity of these proteins (Chmelar et al., 2012), they were also considered to be interesting candidate antigens for anti-tick vaccines.

Thus, the main objective of the present work was to assess the protective efficacy of these salivary proteins, including the identification of potentially protective linear B-cell epitopes. With this aim, these proteins were produced in recombinant form and tested in vaccine trials, both individually and administered jointly to evaluate potentially additive protective effects, and were formulated in different adjuvants to evaluate the most adequate antigen-adjuvant combination.

2. Materials and Methods.

2.1. Ticks and tick material.

The *O. moubata* ticks used in this study came from a colony currently maintained at the laboratory of Animal Parasitology (IRNASA, CSIC, Spain). This colony was established from specimens submitted from the Institute for Animal Health, Pirbright, Surrey, UK. The ticks are fed regularly on rabbits and kept in a culture chamber at 28 ºC, with 85 % relative humidity and a 12 h light-dark cycle.

Tick saliva was collected from unfed adult ticks after stimulating them with 1% pilocarpine, as described previously (Díaz-Martín et al., 2013). Protein concentrations in saliva samples were measured with the Bradford assay (Bio-Rad) and samples were stored at -20 ºC.

Total RNA from the salivary glands of unfed adult ticks (10 males + 10 females) was purified using the NucleoSpin RNA II kit (Macherey-Nagel), following the manufacturer's instructions, and preserved at -80 ºC.

2.2. Cloning, expression and purification of recombinant proteins.

2.2.1. Starting material.

Recombinant plasmids containing the cDNA coding sequences of the proteins of interest were obtained from salivary gland cDNA libraries constructed previously (Manzano-Román et al., 2012).

Plasmids containing the complete cDNA coding sequences of APY and MOU came from the entry library cloned in pDNOR_222 (Invitrogen), while plasmids containing the complete cDNA coding sequences of PLA2, 7DB-like and RP-60S came from the

153 expression library cloned in pANT_GST (DNASU Plasmid Repository). These plasmids
154 were used as templates for amplification of the target sequences (Table 1).
155 For HSP90, the recombinant plasmid available (pANT_GST-HSP90) contained an
156 incomplete cDNA fragment lacking the 5' end. Thus, the missing sequence was first
157 obtained by 5'-RACE from total RNA of salivary glands using the First Choice RLM-RACE
158 kit (Life Technologies) according to the manufacturer's instructions, and two primers
159 that were designed *ad hoc*: GSP2-HSP90 (5'-AATAGATGTGCTTCTGGTTTTCCTTC) and
160 GSP3-HSP90 (5'-ATATTTTTGCTGAACTGCTCATAGAA). Once its 5' end was known, the
161 complete cDNA coding sequence of HSP90 was amplified from total RNA with primer
162 pair Hsp90Kpn55/Hsp90Kpn33 (Table 1). The PCR product was purified and cloned into
163 the pSC-A sequencing vector (Agilent) and its sequence was confirmed following
164 standard procedures described elsewhere (Díaz-Martín et al., 2011). The verified pSC-
165 A-HSP90 construction was used as a template for PCR amplification and the sub-
166 cloning of HSP90 (Table 1).
167 The complete nucleotide sequences coding for these proteins were uploaded
168 into GenBank and the corresponding amino acid sequences were subjected to a basic
169 computational analysis for the identification of restriction enzyme sites, signal
170 peptides, non-classic secretion signals, transmembrane helices and GPI anchor sites,
171 using the appropriate tools available at the Expasy website (http://www.expasy.org/)
172 as reported elsewhere (Díaz-Martín et al., 2013).
173
174 *2.2.2. PCR amplification of target sequences and subcloning into expression*
175 *vectors.*
176 For amplification of the coding cDNAs, six specific primer pairs were designed
177 that included suitable restriction sites to assist in the sub-cloning into the
178 corresponding expression vector. These primer pairs amplified the whole coding
179 sequences except the pairs designed for 7DB-like and APY, which amplified truncated
180 versions, with no signal peptide, of the corresponding protein. All the proteins except
181 MOU were cloned into the pQE-30 expression vector (Qiagen). Owing to its small size
182 (63 amino acids), MOU was cloned into pGEX-4T-1 (Amersham) in order to produce it
183 as a larger fusion protein (MOU-GST) and to improve its immunogenicity. Table 1
184 summarizes all the templates and primers used in the PCR amplifications as well as the

restriction enzymes and expression vectors involved. Table 2 compiles the specific conditions for the different PCRs. The PCR products were purified, digested and cloned into the corresponding expression vector following standard procedures described in previous works (de la Torre et al., 2010; Díaz-Martín et al., 2011).

2.2.3. Protein expression and purification.

Recombinant pQE-30 plasmids containing PLA2, 7DB-like, RP-60S, HSP90 and APY were transformed into *E. coli* M15 cells and protein expression was induced with 1 mM IPTG. Except for HSP90, whose expression failed, all these proteins were expressed in 100 % insoluble form. Thus, they were solubilized with 8 M urea, purified by nickel affinity chromatography in denaturing conditions, and dialyzed against phosphate-buffered saline (PBS), pH 7.4, for 24 h at 4 ºC according to the procedure described by Díaz-Martín et al. (2011).

The recombinant pGEX-4T-1-MOU plasmid was transformed into *E. coli* BL21 cells and protein expression was induced with 0.1 mM IPTG. The MOU-GST fusion protein was expressed in 100 % insoluble form, and was therefore purified from the cellular lysate pellet by electroelution from SDS-PAGE gels following the procedure described by Harlow and Lane (1988).

The concentrations of the purified proteins were measured by spectrophotometry at 280 nm and proteins were stored at -20 ºC.

2.3. Vaccine trials.

2.3.1. Trial 1.

The aim of this trial was to assess the individual capacity of the above recombinant antigens to induce protective responses in rabbits. Each recombinant protein was administered to a group of three rabbits (New Zealand white) in Freund's adjuvant. Two additional groups of rabbits were immunized, either with recombinant GST from *Schistosoma japonicum* (rSjGST; Sigma) in Freund's adjuvant or with the adjuvant alone, and used as controls.

Each animal was vaccinated at 15-day intervals with three doses of 200 µg of protein per dose administered subcutaneously. The first dose was administered

emulsified in Freund's complete adjuvant (FCA); the second in Freund's incomplete adjuvant (FIA), and the third dose with no adjuvant.

Rabbits were bled immediately before the administration of the first dose (pre-immune sera) and at seven days after the third one, immediately before tick infestation (immune sera). Blood samples were allowed to clot and sera were removed and stored at -80 ºC. In the immune sera, the antibody titres to the homologous recombinant protein were tested by ELISA and their reactivity to *O. moubata* saliva was tested by ELISA and Western blot according to standard procedures (García-Varas et al., 2010; Manzano-Román et al., 2015).

At seven days after the third antigen dose, all rabbits were infested with 15 females, 25 males and 50 nymphs-2 of *O. moubata* per rabbit. The parasites were allowed to feed on the rabbits for a maximum of 2 hours after which they were removed from the animals. The degree of protection was determined by measuring the amount of blood ingested (difference in tick weight before feeding and 24 hours after feeding), the oviposition (number of eggs per female) and fertility (number of nymphs-1 per female) rates, the moulting rates of immature stages and the mortality rates of all developmental stages.

The values obtained for the parasites fed on the animals from each group were summarized 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.

Vaccine efficacy (E) was calculated as E = 100 (1- S x F), where S and F respectively represent the reduction in female survival and fertility in ticks fed on vaccinated rabbits as compared to those fed on control rabbits (treated with adjuvant alone or with adjuvant + rSjGST).

2.3.2. Trial 2.

The goal of this trial was to assess the joint vaccine efficacy of PLA2, APY and MOU formulated together in different adjuvants (Freund's or Montanide ISA 50 V2, Seppic) with or without an immune-stimulant (namely, the TLR9 agonist CpG ODN-1826, Miltenyi Biotec).

With this aim, antigen doses containing 100 µg of each recombinant protein in 1 ml of PBS were prepared and administered to different groups of 3 rabbits per group as follows. Group 1: the antigen mixture was supplemented with 100 µg/dose of ODN-1826 and emulsified in 1 ml of Montanide ISA 50 V2. Group 2: the antigen mixture was emulsified in Montanide without ODN-1826. Group 3: the antigen mixture was emulsified in FCA and FIA, respectively, for the first two doses and without adjuvant for the third dose. Each rabbit received three antigen doses subcutaneously at 3-week intervals. An additional group of three rabbits was treated with Montanide ISA 50 V2 plus ODN-1826 and used as control.

Rabbits were bled immediately before the administration of the first dose (pre-immune sera) and at 14 and 28 days after the third one (immune sera), immediately before tick infestations. Blood samples were allowed to clot and sera were removed and stored at -80 ºC. In the immune sera, the antibody titres to each recombinant protein and the reactivity to *O. moubata* saliva were tested by ELISA as described previously (Manzano-Román et al., 2015).

At 14 and 28 days after the third antigen dose, all rabbits were subjected to two tick infestations with 15 females, 25 males and 50 nymphs-3 of *O. moubata* per rabbit and infestation. The tick infestations, the assessment of vaccine effects on the parasites, and the calculation of vaccine efficacies were accomplished as described before for trial 1. Additionally, the vaccine efficacies of each treatment were compared between infestations using Student's T test.

All animal manipulations were performed according to the rules from the Ethical and Animal Welfare Committee of the Institution where the experiments were conducted (IRNASA, CSIC), following the corresponding EU rules and regulations.

2.4. Peptide arrays and epitope mapping (pepscan).

Pepscans were performed on PLA2, APY and MOU to identify the linear B-cell epitopes recognized by the protected animals from trial 1.

With this aim, peptide arrays of PLA2, APY and MOU were synthesized and spotted onto cellulose sheets at the Proteomics Service of the Centro Nacional de Biotecnología (Madrid, Spain). Each sheet contained 2 identical replicas of 88, 192 and 18 overlapping 12-mer peptides with 9 amino acid overlaps, which covered the entire

sequence of the PLA2 (AGJ90343.1), APY (AGJ90350.1) and MOU (AGJ90345.1), respectively (Suppl. Fig. 1).

Pepscans were carried out as already described (Manzano-Román et al., 2015). Before use, the sheets were submerged in ethanol to facilitate the hydration of hydrophobic peptides, rinsed 3 times (10 min each) in 50 mM Tris, 150 mM NaCl (TBS), pH 7, and then blocked in TBS containing 5% skimmed milk, 5% sucrose and 0.05% Tween 20 (blocking solution) overnight at 4 ºC. Then, they were washed in TBS containing 0.05% Tween 20 (washing buffer) and incubated with the corresponding serum pool diluted 1/1,000 in blocking solution for two hours at room temperature (RT). Three serum pools specifically recognizing PLA2, APY and MOU were prepared from the immune sera obtained in vaccine trial 1. After 3 washes, the sheets were incubated with peroxidase-conjugated anti-rabbit IgG diluted 1/10,000 in blocking solution for two hours at RT. After six new washes, the sheets were incubated with Immun-Star WesternC (BIO-RAD) for 5 min at RT.

Negative controls included sheets processed without the primary antibodies or with the pooled pre-immune sera from trial 1, or with the pooled immune sera from animals immunized with rSjGST.

The membranes were scanned with a ChemiDoc device using Image Lab software (BIO-RAD). Image analysis was carried out using Image Master 2D platinum software (GE Healthcare Life sciences), including quantification of the volume of the spots recognized. A peptide was considered positively bound to the antibody when the signal of the corresponding spot was higher than two-fold the average signal of the spots revealed in the negative control membranes. Linear B-cell epitopes were considered only when two or more positive consecutive peptides were recognized in the protein sequence.

2.5. Computational analysis, 3-D modelling and linear B-cell epitope prediction.

The secondary structure and disordered regions of PLA2, APY and MOU were predicted using the Phyre2 (Kelley and Sternberg, 2009), the Disopred2 Disorder prediction (Ward et al., 2004) and the I-Tasser (Zhang, 2008; Roy et al., 2010) servers. For each protein, the three predictions were aligned and the consensus secondary structures were obtained. Three-dimensional (3-D) models of the proteins were

311 generated using the I-Tasser server and the 3-D models were visualised and handled
312 using the PyMol package (DeLano, 2002).
313 For linear B-cell epitope prediction, the BcePred:prediction and the ABCpred
314 Prediction servers were employed using default parameters (Saha and Raghava, 2004,
315 2006).
316
317 **3. Results.**
318
319 *3.1. Nucleotide and amino acid sequences.*
320 Complete cDNA coding sequences of the target proteins were verified and
321 uploaded into GenBank with the accession numbers indicated in table 3. *In silico*
322 translations and analysis of the corresponding amino acid sequences revealed that
323 7DB-like, APY and MOU had signal peptide while PLA2, 7DB-like and APY had non-
324 classical secretion and RP-60S and HSP90 lacked any secretion signal, classical or not.
325 Only MOU had a predicted transmembrane helix and none of them had GPI anchors
326 (Table 3).
327
328 *3.2. Recombinant protein production.*
329 The full-length recombinant forms of PLA2 and RP-60S, the truncated versions of
330 7DB-like and APY, and the MOU-GST fusion protein were all successfully expressed and
331 purified (Fig. 1). All of them migrated in SDS-PAGE gels as a single band of the
332 predicted molecular weight (MW), except 7DB-like, which showed an experimental
333 MW of twice its theoretical MW (24 kDa *vs*. 12.28 kDa). The identity of this
334 recombinant as the predicted 7DB-like was later confirmed by MALDI-TOF/TOF mass
335 spectrometry analysis of the corresponding gel band; this suggests a possible
336 dimerization of this recombinant.
337 Regarding HSP90, after inducing its expression with IPTG the optical density of
338 the cell culture decreased and no recombinant protein was recovered from the cell
339 lysate. Additional attempts to express HSP90 in different cell culture and induction
340 conditions also failed, suggesting cell toxicity. Consequently, this protein was excluded
341 from ensuing experiments.
342

343 *3.3. Vaccination trial 1. Humoral immune response and protective effects induced by*
344 *the individual recombinant antigens.*

345 The immune sera from vaccinated rabbits showed high reactivity to the
346 homologous recombinant antigen, reaching optical densities (ODs) between 0.8 and
347 1.6 and IgG antibody titres higher than 1/12,800. The only exceptions were the sera
348 from rabbits vaccinated with RP-60S, which had ODs around 0.4 and average IgG titres
349 of 1/1,600 (Fig. 2A). When tested against the *O. moubata* saliva, the sera to PLA2, APY
350 and MOU showed medium-low reactivity (OD between 0.2 and 0.4), while the sera to
351 7DB-like and RP-60S showed low reactivity (OD between 0.2 and 0.1), i.e., barely
352 surpassing that of the pre-immune sera (Fig. 2A).

353 According to Western blot, each immune serum recognized a single band of the
354 expected size on its homologous recombinant antigen, excluding the anti-PLA2 sera,
355 which revealed 3 bands of nearly 34, 68 and 100 kDa, suggesting oligomerization of the
356 recombinant PLA2 (Fig. 2B). Furthermore, the sera to PLA2, APY and MOU-GST also
357 recognized their corresponding native antigens on the *O. moubata* saliva (Fig. 2C): anti-
358 APY sera revealed a single band of the expected size, anti-PLA2 sera revealed a single
359 band of 68 kDa (twice the size of the monomeric recombinant) and anti-MOU-GST sera
360 revealed a single band matched in size with GST protein but not MOU (7.2 kDa). By
361 contrast, the sera to 7DB-like and RP-60S did not recognize any band on saliva (Fig. 2C).

362 The protective effect of vaccination was assessed by infesting each rabbit with
363 25 males, 15 females and 50 nymphs-2 of *O. moubata*. No differences were observed
364 between the two control groups (adjuvant, SjGST) for any developmental stage in any
365 of the parameters evaluated. Accordingly, these two groups were merged and treated
366 as a single control group.

367 Protection mainly consisted of decreases in the feeding, reproduction and
368 survival of the ticks fed on vaccinated animals as compared to the ticks fed on controls
369 (Table 4), while unimportant differences were observed in the moulting rate of
370 nymphs between the vaccinated ticks and the controls (not shown).

371 Significant reductions were recorded in the amount of blood ingested by
372 females and nymphs fed on all vaccinated rabbits and by males fed on rabbits
373 vaccinated with APY and MOU. APY induced the strongest reductions in feeding in all
374 developmental stages. Highly significant reductions in female oviposition and fertility

were observed for the rabbits vaccinated with PLA2, APY and MOU, while less significant or non-significant reductions in these parameters were recorded in the rabbits vaccinated with RP-60S and 7DB-like. Regarding mortality, significant increases were recorded for all developmental stages fed on rabbits vaccinated with PLA2, as well as for males fed on rabbits vaccinated with APY and for females and nymphs fed on rabbits vaccinated with RP-60S (Table 4).

Vaccine efficacy (E) was calculated for each recombinant antigen based on the decreases in female survival and fertility, since these parameters are largely responsible for the size of the next tick generation and hence the evolution of the tick population. Here, the most protective antigen was PLA2 (44.2 %), followed by MOU (43.2 %) and APY (27.2 %). The least protective ones were 7DB-like (12 %) and RP-60S (19.9 %), which showed efficacies lower than 20 %, although these efficacies were also statistically significant with respect to the control group (Fig. 3).

3.4. Linear B-cell epitopes recognized on the PLA2, APY and MOU by the vaccine-induced anti-recombinant antibodies.

To map their linear B-cell epitopes, PLA2, APY and MOU peptide arrays were scanned with the sera from rabbits immunized with recombinant PLA2, APY and MOU.

Control sheets processed without primary antibodies did not reveal any spots on any of the arrays (not shown), while the negative pre-immune sera and the sera to SjGST revealed some scattered faint spots on the three peptide arrays, whose volume values served to calculate the threshold for positivity (Suppl. Fig. 1; Suppl. Table 1).

The sera to PLA2, APY and MOU only revealed B-cell linear epitopes on their homologous protein, indicating a lack of cross-reactivity between them (Suppl. Fig. 1; Suppl. Table 1). On PLA2, the antibodies revealed three strongly reactive epitopes and eight epitopes with medium-low reactivity; on APY the antibodies revealed two strong and seven medium-low reactive epitopes, and on MOU the antibodies revealed just two strong overlapping epitopes. For PLA2 and APY, most of their linear B-cell epitopes mapped onto structured regions of proteins (mainly α-helices), while for MOU the two B-cell epitopes mapped onto the disordered central region of the protein (Fig. 4A).

Three dimensional models of PLA2, APY and MOU showed that all the epitopes strongly recognized by the antibodies were located on the surface of the molecules

(Fig. 4B), while the epitopes recognized less intensely were located deeper inside the molecules, where they are probably less accessible to antibodies (not shown).

3.5. Prediction of theoretical linear B-cell epitopes.

For PLA2, the ABCpred and BcePred servers predicted two different sets of 7 and 8 epitopes respectively (Suppl. Fig. 2). The epitopes of each set were distributed throughout the entire PLA2 amino acid sequence and only partly aligned between both predictions, and with the 11 experimental epitopes revealed by the pepscan. For APY a similar result was observed: the servers predicted two different sets of 16 epitopes each, which only partly overlapped between sets, and with the 9 experimental epitopes revealed by the pepscan. By contrast, both servers predicted almost identical epitopes for MOU, which aligned quite faithfully to the experimental epitopes revealed by the antibodies.

3.6. Vaccination trial 2. Humoral immune response and protective effects induced by joint administration of the recombinant antigens.

At 14 days after the third antigen dose (d.p.i.), the immune sera from all vaccinated rabbits showed IgG antibody titres higher than 1/12,800 to each single recombinant. The ODs of all these sera at 1/100 dilution were always close to 1.3 and no significant differences in reactivity to the different recombinant proteins were observed within or between the rabbit groups (Fig. 5). The same was the case of the immune sera obtained at 28 d.p.i. (14 days after the first infestation), although these sera showed ODs slightly higher than their 14 d.p.i. counterparts.

When analysed against saliva, the immune sera from vaccinated rabbits at 14 d.p.i. showed medium reactivity. The most reactive sera were those from rabbits vaccinated with the antigens formulated in Montanide + CpG (average OD, 0.63), followed by the sera from rabbits vaccinated with the antigens formulated in Montanide alone (average OD, 0.46), and finally by the sera from rabbits vaccinated with the antigens formulated in Freund's adjuvant (average OD, 0.37). The sera obtained at 28 d.p.i. (14 days after the first infestation) showed notable increases in their ODs to saliva (28 %, 57% and 84 % for groups 1, 2 and 3, respectively); however, these were not statistically significant because of the variability within each group. As

439 expected, the sera obtained from control rabbits at 14 d.p.i. did not react to saliva but
440 the sera obtained at 28 d.p.i. showed medium-low reactivity (average OD 0.2).

441 The main protective effects observed in this second trial were similar to those
442 of trial 1 and mainly consisted of decreases in feeding, reproduction and survival of the
443 ticks fed on vaccinated animals as compared to the ticks fed on controls. However, in
444 trial 2 these parameters showed higher variation between developmental stages,
445 antigen formulations and infestations (Table 5).

446 In both infestations, the reduction in ingested blood was more significant in
447 females and nymphs than in males. The antigen formulation in Montanide alone and
448 Freund's reduced feeding in females and nymphs more significantly than formulation
449 in Montanide plus CpG. The reduction in survival affected males in the first infestation,
450 and males, females and nymphs in the second infestation to a significant extent. In
451 males, the highest mortality was associated with antigen formulated in Montanide plus
452 CpG, while in females and nymphs it was associated with antigen formulated in
453 Freund's. Female oviposition and fertility rates paralleled each other, showing similar
454 figures for each antigen formulation and infestation. In both infestations, both
455 parameters decreased significantly with the three antigen formulations. In the first
456 infestation, Freund's caused the greatest reduction (close to 50 %), followed by
457 Montanide alone (around 35 %) and Montanide plus CpG (< 20 %). In the second
458 infestation, the inhibition of female reproduction was increased by Montanide plus
459 CpG and Montanide alone (reaching 36 % and 50 %, respectively) while it decreased
460 slightly with Freund's (from 50 % to 40 %) (Table 5).

461 Vaccine efficacy (E) was calculated from the decreases in female survival and
462 fertility for each antigen formulation and infestation (Fig. 6). In both infestations, the
463 antigen formulation in Freund's provided the highest efficacy (50% and 58% in the 1st
464 and 2nd infestation, respectively) followed by Montanide alone (39% and 57%) and
465 Montanide plus CpG (24% and 36%). Interestingly, vaccine efficacy increased in the
466 second infestation for all antigen formulations. Increases were as high as 50 % for
467 Montanide; 46 % for Montanide plus CpG and, 16 % for Freund's. However, none of
468 these increases was statistically significant owing variations among the rabbits within
469 each group.

Finally, joint administration of PLA2, APY and MOU in Freund's provided a higher vaccine efficacy (50 % in the first infestation) than each of these proteins when they were administered individually in the same adjuvant in trial 1. Thus, a 13.6 % increase in efficacy was obtained in trial 2 as compared to the highest individual efficacy reached in trial 1 (44.2 % for PLA2).

4. Discussion.

An effective vaccine against *O. moubata* would be of enormous help in the elimination of this tick from synanthropic environments, thus improving the prevention and control of ASF and TBRF in endemic areas. Developing this vaccine has been the objective of our team for several years and with this in mind we have focused on identifying protective proteins from *O. moubata* that could be expressed as recombinant vaccine antigens.

Targeting salivary exposed antigens is interesting since subsequent tick infestations of immunized animals will likely trigger anamnestic antibody responses and serve as booster shots, making re-vaccination of the host unnecessary (Radulovic et al., 2014). Conversely, salivary exposed antigens may have been protected from the host immune response along the co-evolution of the tick-host interaction, rendering them sparingly or non-immunogenic, as is the case of most of the components of *O. moubata* saliva (Díaz-Martín et al., 2013). Despite this, it has been observed that vaccination with *O. moubata* salivary anti-haemostatic/anti-inflammatory antigens (i.e., Om44) formulated in Freund's adjuvants favours antigen immunogenicity and provides protective responses, demonstrating that these molecules are suitable targets for anti-tick vaccines (Díaz-Martín et al., 2015).

As mentioned in the introduction, the construction of protein arrays from *O. moubata* salivary glands allowed us to identify several novel salivary anti-haemostatics (PLA2, APY and MOU) as well as some salivary antigens that were recognized by the serum of Om44-vaccinated and protected animals (PLA2, 7DB-like, RP-60S and HSP90); accordingly, all these salivary components are potential targets for vaccines (Manzano-Román et al., 2012).

The secreted salivary PLA2 from *O. moubata* acts as an antagonist ligand for host P-selectin most likely preventing the host haemostatic and inflammatory response

triggered at the tick bite lesion (García-Varas et al., 2010; Manzano-Román et al., 2012). P-selectin expressed on activated endothelial cells and platelets interacts with its receptor on leucocytes, the PSLG-1 molecule. This interaction leads to increased thrombin generation at the site of injury and simultaneously stabilizes the platelet plug (Polgar et al., 2005; Cleator et al., 2006; Zarbock et al., 2007). Since this response could prevent tick feeding, blocking the P-selectin/PSGL-1 interaction by PLA2 at the tick bite site seems to be critical for *O. moubata* to complete its feeding. APY is an orthologue of the *O. savignyi* apyrase, which acts as a platelet aggregation inhibitor by hydrolysing ADP (Stutzer et al., 2009). MOU is an orthologue of the *O. savignyi* savignyigrin, which is an antagonist ligand for the aIIbβ3 integrin (or disintegrin) and inhibits platelet aggregation by a number of agonists including ADP. It blocks binding of integrin aIIbβ3 on the activated platelets to fibrinogen (Mans et al., 2002). Therefore, blocking the function of PLA2, APY and MOU by vaccine-induced antibodies could impact the tick blood feeding process.

Regarding 7DB-like, RP-60S and HSP90, their functions in *O. moubata* saliva are unknown, although some recent work has reported anti-haemostatic, anti-inflammatory and immune-modulatory functions for these kinds of proteins in other organisms, lending further support to their potential value as vaccine targets. Salivary secreted cysteine-rich proteins with 7 disulphide bonds similar to the argasid 7DB protein family (Francischetti et al., 2008a, 2008b; Mans et al., 2008) have been found in ixodid tick saliva, where they seem to be involved in mediating tick anti-haemostatic and anti-complement functions, and RNAi gene-silencing experiments suggest that these proteins could represent interesting vaccine targets (Mulenga et al., 2013; Tirloni et al., 2014). Thus, it is possible that argasid 7DB and 7DB-like proteins could also play similar anti-defensive functions, also representing potential vaccine targets. RP-60S and HSP90 are intracellular housekeeping proteins, but accumulating evidence indicates that in spite of the lack of secretion signals these proteins are actually secreted to tick saliva (Díaz-Martín et al., 2013; Radulovic et al., 2014; Tirloni et al., 2014). In addition, recent studies have described extracellular functions for secreted ribosomal proteins and heat shock proteins as anti-inflammatory agents in mammals and parasites (Pockley, 2003; Lv et al., 2013; Poddar et al., 2013; Vélez et al., 2013;). Accordingly, it is conceivable that the secretion of this kind of protein to tick saliva

534 could be part of the system used by ticks to evade the host inflammatory response to
535 tick feeding, making them candidate antigens to interfere with vaccine-induced
536 antibodies (Radulovic et al., 2014).

537 To evaluate the usefulness of the above-mentioned salivary proteins as vaccine
538 targets, our first objective was to demonstrate their immunogenicity when inoculated
539 as recombinant antigens in rabbits. To do so, the successfully obtained recombinant
540 proteins (all except HSP90, which was toxic for *E. coli*) were administered individually
541 to the animals in Freund's adjuvants. All of them except RP-60S demonstrated strong
542 immunogenicity since they induced the synthesis of high titres of specific antibodies to
543 the homologous recombinant (Fig. 2A), mainly directed to immunodominant epitopes
544 located on the surface of the molecules (Fig. 4B).

545 The sera to PLA2 and APY reacted against their homologous native proteins in
546 saliva, confirming previous evidence of their secretion to this fluid. Regarding PLA2, the
547 specific antibodies revealed three bands of 38, 68 and 100 kDa on the recombinant
548 protein and only recognized the 68 kDa band on the saliva. These results suggest the
549 presence of monomeric and oligomeric forms of this protein, this protein being
550 secreted as a homo-dimer in native saliva.

551 The only band revealed on saliva by the anti-MOU-GST antibodies was
552 compatible in size with that of GST, indicating the presence of GST in *O. moubata*
553 saliva, while the small size of mougrin probably prevented its detection under the
554 electrophoretic conditions used (Tris-Glycine), making it necessary to apply specific
555 conditions to achieve its optimal resolution (Goetz et al., 2004; Schägger, 2006). This is
556 the first report of GST in argasid tick saliva, although GST (and other anti-oxidant
557 proteins) has been already found in the saliva of ixodid ticks, where it is assumed to
558 protect the tick, tick-borne pathogens, and even host tissues from the oxidative stress
559 response to tick-feeding injury (Radulovic et al., 2014). Tick GST has been studied as a
560 target anti-tick vaccine candidate and seems to confer protection in *Rhipicephalus*
561 *microplus* and *Haemaphysalis longicornis* (Parizi et al., 2011). Here, the recognition of
562 *O. moubata* salivary GST by the anti-MOU-SjGST antibodies does not seem to
563 contribute to the protective effects induced by the MOU-SjGST fusion protein since
564 control rabbits vaccinated with SjGST also recognized the *O. moubata* salivary GST (not
565 shown) and did not display any protection (Table 4).

566 The sera to 7DB-like and RB-60S proteins showed very low reactivity against the
567 *O. moubata* saliva in ELISA and they did not recognize their homologous native protein
568 in Western blots (Fig. 2B). At first sight, these results seem to suggest a lack of
569 secretion of these two proteins to saliva. However, the significant reduction in the
570 blood ingested by the specimens fed on the animals vaccinated with these proteins
571 does not support this idea (Table 4); instead the low immunogenicity of RP-60S and/or
572 post-translational modifications and differences in epitope accessibility between the
573 native and the recombinant versions of these proteins could account for this apparent
574 lack of reactivity (Baker et al., 2010).

575 Regarding the protection achieved, the five recombinants induced qualitatively
576 similar protective responses, which mainly consisted of significant reductions in tick
577 feeding performance, strongly suggesting that all of them would be acting as anti-
578 haemostatics to facilitate tick feeding. The reduction in ingested blood and consequent
579 reduction in nutrient availability was later reflected in significant reductions in female
580 oviposition and fertility, especially for PLA2, APY and MOU. In addition, PLA2 induced
581 significant reductions in the survival rate of all the tick developmental stages,
582 suggesting the involvement of this protein in vital processes for ticks, including the
583 production of lipid mediators, the regulation of membrane remodelling, the
584 degradation of foreign phospholipids, and the lysis of host blood cells in the gut lumen
585 (Murakami et al., 2015). Therefore, it could be speculated that this PLA2 and/or other
586 PLA2 isoforms containing cross-reactive epitopes would also be expressed in the tick
587 gut and even in tick reproductive organs. Evidently, this needs further confirmation.

588 In sum, PLA2, APY and MOU showed the highest vaccine efficacies and were
589 considered the best candidates for vaccines, and were therefore subjected to epitope
590 mapping and re-evaluation as a multicomponent vaccine.

591 In most cases, the recognition of a particular epitope by specific antibodies on
592 the antigenic protein is a key event for the immune response to be able to provide
593 protection (Wang et al., 2011; Sharon et al., 2014; Manzano-Román et al., 2015). In
594 agreement with Sharon et al. (2014), our current results strongly suggest that, at least
595 for PLA2, APY and MOU, specific antibodies with high affinity were induced, targeting
596 immunodominant, easily accessible protective epitopes, which most probably interact
597 with host receptors to allow feeding (Fig. 4B). Thus, these epitopes could be included

598 in the current repertory of target candidates for anti-soft tick vaccines and could even
599 serve as a starting point in the search for conserved or similar protective epitopes on
600 functional orthologues in ixodids that would facilitate the development of broader-
601 spectrum anti-tick vaccines (Diaz-Martín et al., 2015).

602 Taken together, the present results confirm that, when administered
603 individually, soft tick salivary antigens are able to induce partial protective immune
604 responses. Such protection mainly consists of impaired tick feeding, most likely as a
605 consequence of the antibody-mediated loss of function of the target antigen at the
606 tick-host interface. Although the exploration of additional adjuvants and immunization
607 protocols could increase the protective efficacy of these antigens, a major factor
608 limiting the vaccine efficacy of individual salivary antigens is the functional redundancy
609 of tick saliva composition, which could compensate the loss of function of the targeted
610 antigen (Chmelar et al., 2012; Kazimírová and Štibrániová, 2013; Wikel, 2013).
611 Accordingly, the use of vaccines formulated with several target antigens would likely
612 increase their protective efficacy (Imamura et al., 2008; Willadsen, 2008b).

613 In this sense, the functional relationship between PLA2, APY and MOU (mainly as
614 anti-platelet aggregation agents) suggested that vaccination with a combination of
615 these proteins could result in a potentially synergistic protective effect. We therefore
616 tested this hypothesis in vaccine trial 2 by administering these three antigens jointly,
617 formulated in two water-in-oil adjuvants, Freund´s and Montanide, which promote
618 prolonged and sustained high antibody titres (Leroux-Roels, 2010; Awate et al., 2013).
619 In addition, we included a third rabbit group in which Montanide was used in
620 combination with the immunostimulant CpG ODN-1826, which is a TLR9 agonist that
621 potentiates humoral responses (Klinman, 2004; Liu et al., 2012, Scheiermann and
622 Klinman, 2014).

623 The results of trial 2 revealed three general trends. First, the administration of
624 the multicomponent vaccine in Freund's increased vaccine efficacy by 13.6% as
625 compared to the best efficacy of the individual antigens, indicating some additive
626 protective effects rather than a real synergistic effect. Immunological interactions and
627 suppression mechanisms by immunodominant antigens causing
628 suppression/modification of the immune response to co-administered antigens have
629 been described (Insel, 1995). These might have limited more potent additive effects in

630 the current vaccine trial with these three anti-haemostatics. However, the high
631 antibody titres to each recombinant antigen used (Fig. 5) does not seem to support
632 this idea; instead they suggest the persistence of additional tick salivary anti-
633 haemostatics untargeted by the vaccine.

634 Second, the administration of ODN-1826 seemed to favour the synthesis of
635 antibodies recognizing the native antigens in saliva (Fig. 5), but it did not enhance
636 vaccine efficacy (Fig. 6). This increased humoral response could be important for later
637 antigen challenges (Scheiermann and Klinman, 2014), but here this effect was not
638 observed as regards vaccine efficacy, which remained below the vaccine efficacy
639 attained with Montanide alone -and Freund's- in both infestations (Fig. 6).

640 Third, the higher levels of specific antibodies and vaccine efficacies achieved
641 after tick infestation challenges clearly indicated that the native antigens inoculated
642 with tick saliva during natural contact acted as boosting antigen doses. This notable
643 effect is interesting since it confirms previous observations that "silent" salivary
644 exposed antigens from ticks can be rendered immunogenic by vaccination (Kotsyfakis
645 et al., 2008) and that the vaccine-induced protective immune response is enhanced by
646 subsequent tick infestations. The increase in vaccine efficacy observed in the second
647 infestation was not attributed to a vaccine-independent acquired immunity upon tick
648 infestation since natural contacts with *O. moubata* does not induce protective
649 responses in the hosts. This had been observed for rabbits and swine in previous works
650 (Astigarraga et al., 1995; García-Varas et al., 2010) and can be also observed for control
651 rabbits in the current vaccine trial. In these rabbits, the immune response induced by
652 ticks upon the first infestation (Fig. 5) did not resulted in any protection in the second
653 infestation (Table 5).

654 Finally, the high average increase in vaccine efficacy observed in the group
655 vaccinated with Montanide alone suggests that this would be the most convenient
656 adjuvant for the formulation of these antigens.

657 In sum, it appears that a combination of recombinant multi-epitope antigens
658 targeting redundant tick anti-defensive mechanisms could have an important impact
659 on vaccine efficacy. The protection appears to be initiated by the vaccine antigens and
660 is maintained and even amplified by native proteins during subsequent infestations,

661 although for the time being achieving synergistic protective effects does not seem very
662 feasible.
663
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849

850 **Figure captions.**

851

852 **Figure 1.** Expression and purification of recombinant phospholipase A_2 (A), 7DB family
853 member (B), riboprotein 60S-L10 (C), apyrase (D) and fusion protein MOU-GST (E).
854 Coomassie blue-stained 12% SDS-PAGE gels showing supernatants (S) and pellets (P)
855 from cell lysates before (- IPTG) and after (+ IPTG) the induction of protein expression
856 with IPTG. Panels A to D also include the supernatant and pellet after cell lysate pellet
857 solubilisation with 8M urea. Rightmost lane in each panel shows the purified
858 recombinant protein. MW, molecular weight marker including values in kDa.

859

860 **Figure 2.** Antibody response induced by each recombinant protein in the vaccinated
861 rabbits. (A) Reactivity in ELISA of the anti-recombinant antibodies to both the
862 homologous recombinant and the *O. moubata* saliva. Values are the mean OD ± SD at
863 492 nm ($OD_{492\ nm}$ immune sera − $OD_{492\ nm}$ pre-immune sera) from each rabbit group.
864 (B) and (C) Western blot: antigenic bands recognized by the anti-recombinant
865 antibodies on the corresponding recombinant protein and *O. moubata* saliva. Numbers
866 on the left indicate the molecular weight standards. PLA2, phospholipase A_2; 7DB-like,
867 putative 7DB family member; RP-60S, riboprotein 60S L10; APY, apyrase; MOU-GST,
868 mougrin-GST fusion protein.

869

870 **Figure 3.** Trial 1. Vaccine efficacy of the different recombinant antigens calculated as E
871 = 100 (1 - S × F), where S and F represent the reduction in survival and fertility of
872 females fed on vaccinated rabbits as compared to the controls fed on adjuvant and
873 SjGST-treated rabbits. PLA2, phospholipase A_2; 7DB-like, putative 7DB family member;
874 RP-60S, riboprotein 60S L10; APY, apyrase; MOU, mougrin.

875

876 **Figure 4**. **(A)** Amino acid sequences of *O. moubata* PLA2, APY and MOU showing the
877 alpha helices (yellow) and beta sheets (blue) predicted by the Phyre[2], the Disopred2
878 Disorder prediction and the I-tasser servers. The linear B-cell epitopes recognized on
879 each protein by the homologous anti-recombinant antibodies are underlined: thin lines
880 represent faint antibody binding and thick lines strong antibody binding (see Suppl.
881 Table 1 for quantification values). In PLA2 and APY, most epitopes map onto α-helices,

while in MOU both epitopes map onto the disordered/unstructured region of the protein. **(B)** Three-dimensional models of *O. moubata* PLA2, APY and MOU respectively showing the 3, 2 and 2 linear B-cell epitopes strongly recognized by the anti-recombinant antibodies in homologous combination. The amino acid sequences and positions of its epitopes are indicated below each protein. Each colour represents a different epitope, except for MOU, where magenta represents the amino acids shared by the two overlapping epitopes identified in this protein. In the three 3-D models the epitopes localize on the surface of the proteins, where they would be easily accessible to antibodies.

Figure 5. Reactivity in ELISA to PLA2, APY, MOU and *O. moubata* saliva of the immune sera from rabbits vaccinated in trial 2. Sera were obtained at 14 and 28 days after the third antigen dose (d.p.i.). Values are the mean OD ± SD at 492 nm ($OD_{492\ nm}$ immune sera − $OD_{492\ nm}$ pre-immune sera) from each rabbit group. The control group was treated with Montanide ISA 50 V2 and CpG ODN-1826; Ag-Mont-CpG, antigen emulsified in Montanide ISA 50 V2 with CpG ODN-1826; Ag-Mont, antigen emulsified in Montanide alone; Ag-Freund, antigen emulsified in Freund's alone.

Figure 6. Trial 2. Vaccine efficacy of the different formulations in both infestations was calculated as $E = 100 (1 - S \times F)$, where S and F represent the reduction in survival and fertility of females fed on vaccinated rabbits as compared to those fed on control rabbits treated with Montanide plus CpG. Ag-Mont-CpG, antigen emulsified in Montanide ISA 50 V2 with CpG ODN-1826; Ag-Mont, antigen emulsified in Montanide alone; Ag-Freund, antigen emulsified in Freund's alone.

Table 1

Table 1. Recombinant plasmids (templates) and primers used for amplification of the cDNAs coding for the target proteins. Primers include suitable restriction sites (underlined) to assist in the subcloning into the corresponding expression vector (last column) as well as additional nucleotides in 5' to increase the activity of the restriction enzymes involved. (t), proteins for which a truncated version without signal peptide was amplified; PLA2, phospholipase A_2; 7DB-like, putative 7DB family member; RP-60S, riboprotein 60S L10; HSP90, heat shock protein 90; APY, apyrase; MOU, mougrin.

Target protein	Template plasmids	Primer name	Primer sequence	Restriction enzyme	Expression vector
PLA2	Clones in pANT7_GST	PLA2_KpnI55	5'-TTCGGTACCATGACTAAAGAAAACCAGACC	*Kpn*I	pQE-30
		PLA2_KpnI33	5'-TTCGGTACC AGTCATTTTGTCGTCTTTATTG		
7DB-like (t)		tr7DB_KpnI55	5'-TTCGGTACCACTTTCATCATAGAAGATCTTCC		
		7DB_KpnI33	5'-TTCGGTACCTCACGGCGACGCATTTGTCAC		
RP-60S		Rib_KpnI55	5'-TTCGGTACCATGGGTCGCCGTCCGGCAAG		
		Rib_KpnI3	5'-TTCGGTACCTCAGAGGTCCGTGGCCGCTGT		
HSP90	pSC-A-HSP90	Hsp90Kpn55	5'-TTCGGTACCATGCCAGAAGAAGCTCAAATG	*Kpn*I	
		Hsp90Kpn33	5'-TTCGGTACCTTAGTCTACTTCCTCCATGC		
APY (t)	Clones in pDONR_222	trApi55Kpn	5'-TTCGGTACC AAGCCTGCAACGACTCCG		
		Api33Kpn	5'-TTCGGTACCTTAGACCCAGATCTCTTGCTT		
MOU		MougrinEcoRI55	5'-CCGGAATTCATGCAGGCGAAAATCTTGGT	*Eco*RI	pGEX-4T-1
		MougrinEcoRI33	5'-CCGGAATTC TTACCATTTTTCTCCGCATAG		

Table 2. PCR conditions for amplification of cDNAs coding for the target proteins. (t), proteins for which a truncated version without signal peptide was amplified; PLA2, phospholipase A_2; 7DB-like, putative 7DB family member; RP-60S, riboprotein 60S L10; HSP90, heat shock protein 90; APY, apyrase; MOU, mougrin.

Target protein	PCR conditions		
PLA2	2 min 94 °C	5 cycles (94 °C 15 s, 56 °C 30 s, 72 °C 40 s) + 30 cycles (94 °C 15 s, 62 °C 30 s, 72 °C 40 s)	7 min 72 °C
7DB-like (t)	2 min 94 °C	5 cycles (94 °C 15 s, 60 °C 30 s, 72 °C 40 s) + 30 cycles (94 °C 15 s, 66 °C 30 s, 72 °C 40 s)	7 min 72 °C
RP-60S	2 min 94 °C	5 cycles (94 °C 15 s, 66 °C 30 s, 72 °C 40 s) + 35 cycles (94 °C 15 s, 72 °C 30 s, 72 °C 90 s)	7 min 72 °C
HSP90	2 min 94 °C	5 cycles (94 °C 15 s, 56 °C 30 s, 72 °C 40 s) + 30 cycles (94 °C 15 s, 60 °C 30 s, 72 °C 40 s)	7 min 72 °C
APY (t)	2 min 94 °C	5 cycles (94 °C 15 s, 56 °C 30 s, 72 °C 40 s) + 30 cycles (94 °C 15 s, 62 °C 30 s, 72 °C 40 s)	7 min 72 °C
MOU	2 min 94 °C	5 cycles (94 °C 15 s, 56 °C 30 s, 72 °C 40 s) + 30 cycles (94 °C 15 s, 62 °C 30 s, 72 °C 40 s)	7 min 72 °C

Table 3. Nucleotide sequences coding for the target proteins: GenBank accession number and size in base pairs (bp). Protein sequences: size in number of amino acid (aa), theoretical isoelectric point (pI) and molecular weight (MW) and predicted presence/absence of signal peptide, transmembrane helices (TH), non-classical secretion signals and GPI anchors. PLA2, phospholipase A_2; 7DB-like, putative 7DB family member; RP-60S, riboprotein 60S L10; HSP90, heat shock protein 90; APY, apyrase; MOU, mougrin.

	Nucleotide sequence		**Amino acid sequence**						
Protein name	**GenBank accession no.**	**Size (bp)**	**Size (aa)**	**pI**	**MW (kDa)**	**Signal peptide (aa)**	**TH**	**Non-classical secretion signal**	**GPI anchor**
PLA2	KC908103.1	816	271	5.71	31.44	no	no	yes	no
7DB-like	KC908104.1	342	113	4.22	12.38	Yes (1-17)	no	yes	no
RP-60S	KC908109.1	624	207	10.66	23.56	no	no	no	no
HSP90	KC908107.1	1692	563	5.03	65.73	no	no	no	no
APY	KC908110.1	1755	584	8.69	65.23	Yes (1-18)	no	yes	no
MOU	KC908105.1	192	63	4.64	7.21	Yes (1-21)	yes	no	no

Table 4

Table 4. Effect of the individual vaccination with each recombinant antigen on *O. moubata* specimens fed on control and vaccinated rabbits. Results are shown as mean ± standard deviation for each rabbit group. Means were compared between ticks fed on vaccinated and control rabbits (merged adjuvant-treated and SjGST-vaccinated 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 merged controls. * $p < 0.05$; ** $p < 0.01$. SjGST, *Schistosoma japonicum* glutathione-S transferase; PLA2, phospholipase A_2; 7DB-like, putative 7DB family member; RP-60S, riboprotein 60S L10; APY, apyrase; MOU-GST, mougrin-GST fusion protein.

Parameter	Developmental stage	Merged control (adjuvant and SjGST, n = 6)	Control rabbits (adjuvant)	Control rabbits (SjGST)	PLA2 vaccinated rabbits (% reduction)	7DB-like vaccinated rabbits (% reduction)	RP-60S vaccinated rabbits (% reduction)	APY vaccinated rabbits (% reduction)	MOU-GST vaccinated rabbits (% reduction)
Ingested blood (mg)	Males	28.1 ± 4.3	27.8 ± 4.3	28.4 ± 5.1	27.2 ± 3.2 (3.2)	27.2 ± 6.5 (3.1)	25.3 ± 0.3 (10.2)	19.6 ± 1.3 (30.5)**	19.7 ± 2.7 (30.0)**
	Females	233.1 ± 16.5	239.8 ± 17.6	219.7 ± 6.3	186.4 ± 19.2 (20.1)**	173.3 ± 26.4 (25.6)**	204.4 ± 14.5 (12.3)**	172.2 ± 5.7 (26.1)**	185.4 ± 18.2 (20.5)**
	Nymphs-2	26.3 ± 3.1	26.4 ± 3.1	26.3 ± 4	21.6 ± 2.5 (18.1)**	21.5 ± 0.9 (18.6)**	21.4 ± 1.6 (18.8)**	15.9 ± 2.1 (39.7)**	19.9 ± 4.3 (24.2)**
Mortality (%)	Males	4.3 ± 3.6	6 ± 2	2.6 ± 4.1	18.6 ± 3.9 (14.3)**	8 ± 0 (3.7)	10.7 ± 3.9 (6.4)	16 ± 4.4 (11.7)**	5.3 ± 3.9 (1)
	Females	5 ± 3	5.6 ± 2.7	4.4 ± 3.4	31.6 ± 21.1 (26.6)**	4.4 ± 3.3 (0)	15.6 ± 11.7 (10.6)*	2.2 ± 3.2 (0)	8.9 ± 3.2 (3.9)
	Nymphs-2	2.7 ± 3.4	5.3 ± 3	0 ± 0	11.3 ± 7.9 (8.4)**	6 ± 2.9 (3.3)	12 ± 1.7 (9.3)**	4 ± 0 (1.3)	2.7 ± 3.9 (0)
Oviposition (no. eggs/female)	Females	209.3 ± 14.9	205.3 ± 19.8	213.3 ± 7.9	160.5 ± 36.8 (23.3)**	192.9 ± 5.9 (8.7)	181.7 ± 2 (13.2)*	153.6 ± 6.1 (26.6)**	142.5 ± 8.9 (31.9)**
Fertility (no. nymphs/female)	Females	189.1 ± 14.3	191.3 ± 20	186.8 ± 4.7	146.3 ± 32 (22.6)**	168.9 ± 10.1 (10.7)*	171.9 ± 5 (9.1)	140.3 ± 8,6 (25.8)**	116.9 ± 25.2 (38.2)**

Table 5

Table 5. Effect of the vaccination with the three recombinant antigens administered together in different formulations on *O. moubata* specimens fed on control and vaccinated rabbits in two infestations performed 14 days apart. For each infestation, results are shown as mean ± 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. * $p < 0.05$; ** $p < 0.01$. Control group was treated with Montanide ISA 50 V2 and CpG ODN-1826, without antigen. Group 1 was immunized with the three recombinants (PLA2, APY and MOU) emulsified in Montanide ISA 50 V2 with CpG ODN-1826; group 2 was immunized with the three recombinants emulsified in Montanide ISA 50 V2, and group 3 was immunized with the three recombinants emulsified in Freund's.

		First infestation				Second infestation			
Parameter	**Developmental stage**	**Control**	**Group 1 Ag-Mont-CpG (% reduction)**	**Group 2 Ag-Montanide (% reduction)**	**Group 3 Ag-Freund's (% reduction)**	**Control**	**Group 1 Ag-Mont-CpG (% reduction)**	**Group 2 Ag-Montanide (% reduction)**	**Group 3 Ag-Freund's (% reduction)**
Ingested blood (mg)	Males	23.1 ± 1.8	22.1 ± 5.1 (4.7)	21.1 ± 5.4 (8.8)	22.1 ± 1.8 (4.7)	23.3 ± 2.7	18.8 ± 3.7 (19.3)	22.7 ± 0.8 (2.8)	17.2 ± 11.7 (26.1)
	Females	196.4 ± 14.5	193.3 ± 24.8 (1.6)	150.8 ± 17.9 (23.2)**	154.4 ± 12 (21.4)**	243.9 ± 12.8	237.5 ± 33.2 (2.6)	171.2 ± 5.9 (29.8)**	197.4 ± 23.6 (19)*
	Nymphs-3	47.2 ± 2.3	34.7 ± 27.5 (26.4)*	31.9 ± 1.7 (32.5)**	36.3 ± 6.1 (23.2)*	47.3 ± 1.7	46.9 ± 1.4 (1)	41.8 ± 1.8 (11.7)	39.1 ± 5.2 (17.3)**
Mortality (%)	Males	5.3 ± 2.3	18.7 ± 7.4 (13.4)**	16 ± 4.4 (10.7)**	5.3 ± 1.9 (0)	4 ± 4	16.1 ± 3.8 (12.1)**	6 ± 2.2 (2)	10.7 ± 2.0 (6.7)**
	Females	7.1 ± 0.8	15.5 ± 3.4 (8.4)	13.3 ± 0.0 (6.2)	8.9 ± 8.7 (1.8)	4.4 ± 3.8	20 ± 10.3 (15.6)	16.7 ± 3.7 (12.3)	31.1 ± 13.1 (26.7)**
	Nymphs-3	2.7 ± 1.1	1.3 ± 2.1 (0)	1.1 ± 1.1 (0)	4.7 ± 3.6 (2)	10.7 ± 8.1	10 ± 4.7 (0)	13 ± 5.4 (2.3)	24.7 ± 9 (14)**
Oviposition (no. eggs/female)	Females	222.3 ± 18.6	184.6 ± 18.2 (17.0)**	144.9 ± 2.3 (34.8)**	116.7 ± 4 (47.5)**	221.2 ± 25.4	155.9 ± 27.3 (26.2)*	113.6 ± 28.7 (46.2)**	126.3 ± 33.7 (40.2)*
Fertility (no. nymphs/female)	Females	190.8 ± 9.9	159.9 ± 8.1 (16.2)**	125.5 ± 9.2 (34.2)**	100.7 ± 3.8 (47.2)**	187.3 ± 19.2	140.1 ± 21.9 (25.2)*	91.1 ± 39.5 (51.4)**	110.5 ± 28.8 (41)**

Figure 1.

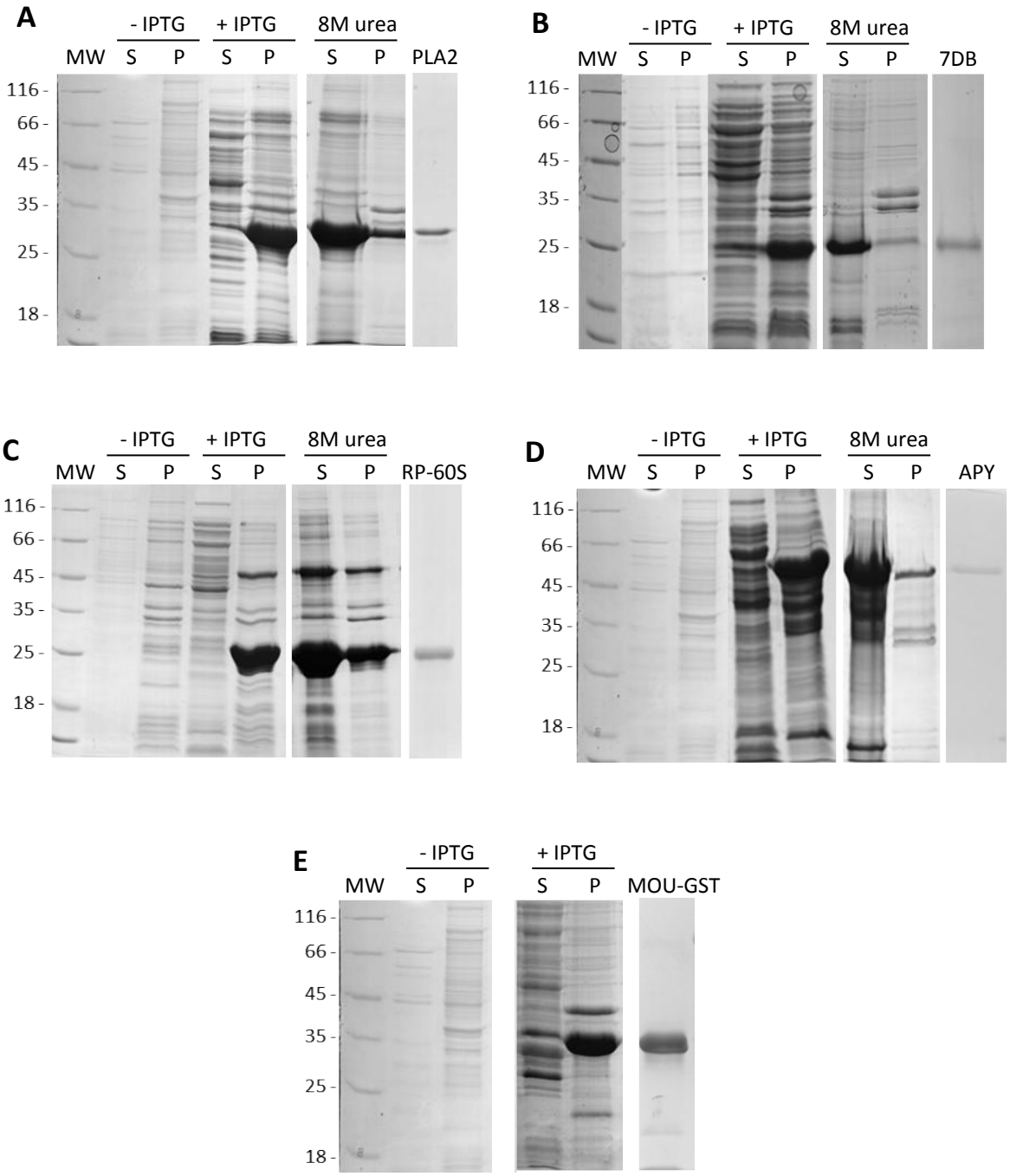
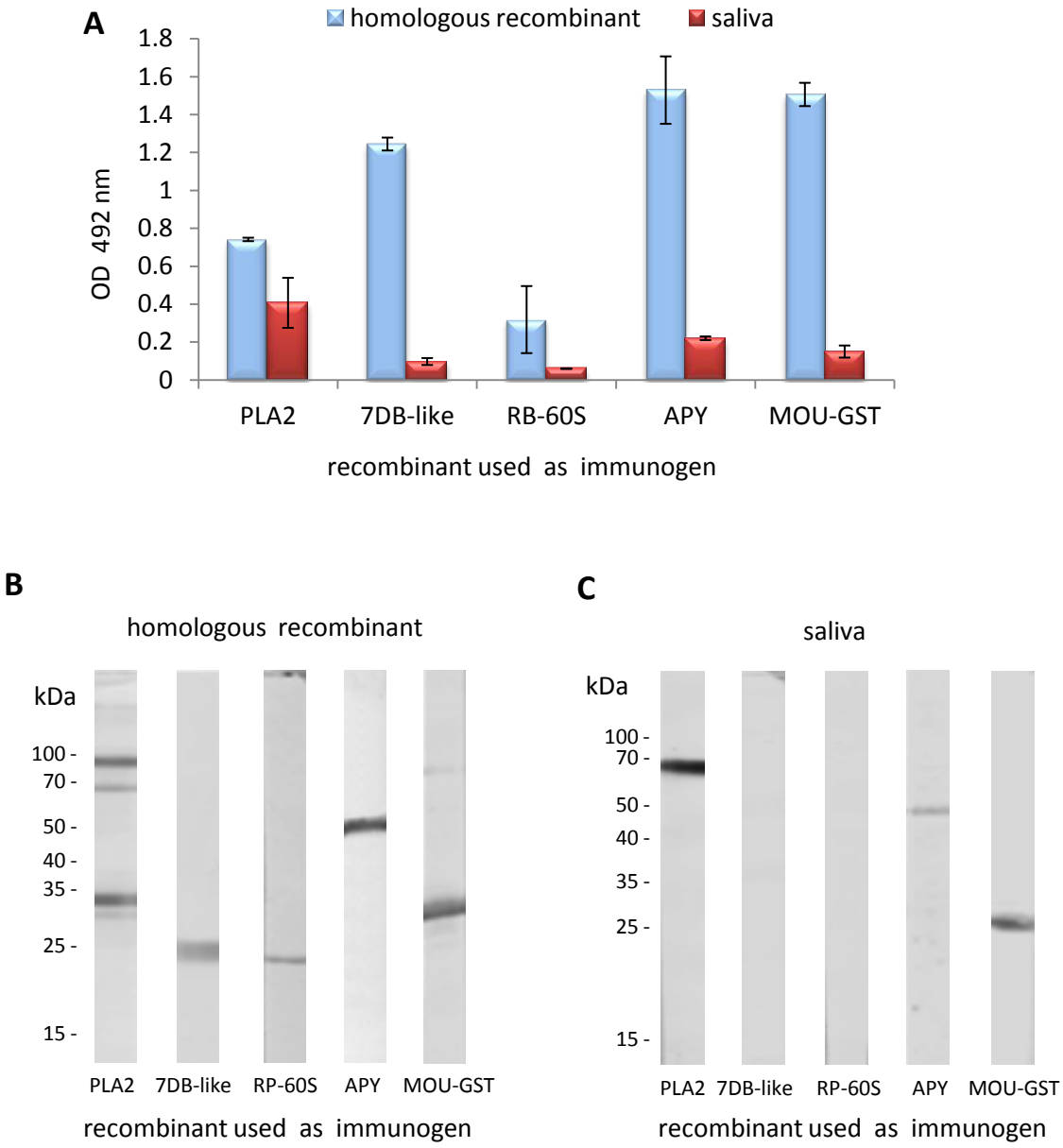

A
- IPTG
+ IPTG
8M urea
MW
S
P
S
P
S
P
PLA2
116
66
45
35
25
18
B
- IPTG
+ IPTG
8M urea
MW
S
P
S
P
S
P
7DB
116
66
45
35
25
18
C
- IPTG
+ IPTG
8M urea
MW
S
P
S
P
S
P
RP-60S
116
66
45
35
25
18
D
- IPTG
+ IPTG
8M urea
MW
S
P
S
P
S
P
APY
116
66
45
35
25
18
E
- IPTG
+ IPTG
MW
S
P
S
P
MOU-GST
116
66
45
35
25
18

Figure 2.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homologous recombinant
saliva
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1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
PLA2
7DB-like
RB-60S
APY
MOU-GST
recombinant used as immunogen
B
homologous recombinant
kDa
100 -
70 -
50 -
40 -
35 -
25 -
15 -
PLA2
7DB-like
RP-60S
APY
MOU-GST
recombinant used as immunogen
C
saliva
kDa
100 -
70 -
50 -
40 -
35 -
25 -
15 -
PLA2
7DB-like
RP-60S
APY
MOU-GST
recombinant used as immunogen

Figure 3.

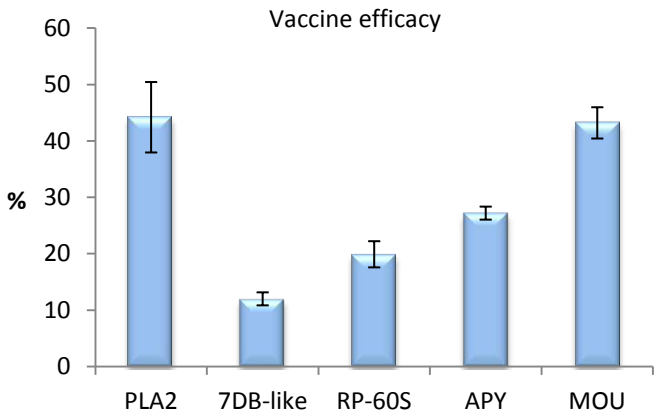
Vaccine efficacy
60
50
40
%
30
20
10
0
PLA2
7DB-like
RP-60S
APY
MOU

Figure 4A.

PLA2 (AGJ90343.1)

```
001 MTKENQTLVEQYLKCEDLGNSVEGKMMSLLDRFGSAVTLATGNTFATQNQIDAADEYVRN
061 CTWHPLMMRTPRDVLLLRWNKNGDYIYPGTKWCGAGNKSERTGDYGTNNETDKCCEAHDN
121 ATDYMLSRSYNANRTMWNPKYYTVTNCADDAKLFDCLLKANTSGSLEFGQAFFDALQVPC
181 FANTYKRDCQSWWDGFAYRYPPKCKPWNKTAEKNWTLIDPPNFYYTFLRVNHNDTYEAAN
241 DIYSWKEVCKLDESLNCWNYTWLNSSIKTTK
```

APY (AGJ90350.1)

```
001 MLKHFFLAFSLLLAVSNAKPATTPKPKCPKKAPDGFTLTILHTNDIHSHFDESNQWGGPC
061 VPKNNKTDHCVAGVTRLATLVKKMKKEYPKALFMNAGDFYQGSVWYTVLKDRIVSAVMKE
121 LKYNAVSLGNHEFDDGPDGLAPFLGNMSEAGVKVIATNVDTKDEPILKDKVLLKSHTFCV
181 EGRRVGVIGAVTEETRTIAKPGNALIKDVIPSLQEEAKRLKAKGVEIIVTITHTGYDVDP
241 YIVGNITELDILVGGHTNTFLYNGTPPTKDKVEGQYPTVVERPDGSRGLIVQDFWFGKYL
301 GFLQVTFDSKGNVNSWQGNPIFVNHSYEEDESMKKPLEPFREIVNEAGRRPIGSSKVVLS
361 ADNKTCRLNECNMMNMVTDSFLAYYADQDSPETMWSNVAAAVVNSGFARSSLPKSDKLTM
421 FDIMRALPYESSLVVLTLKGTDLRKMFEHSVAQFTVTADPRGEFLAVSGMKVKYDLKKPA
481 NKRVVYLRILCTQCVVPRYEVVKNNQTYKIATTSYIANGGDGFEFDKEVIKETKGVVDSE
541 VYLPYIMKMSPLKTAVEGRIFIRNYPKPAISSRYDMSWKQEIWV
```

MOU (AGJ90345.1)

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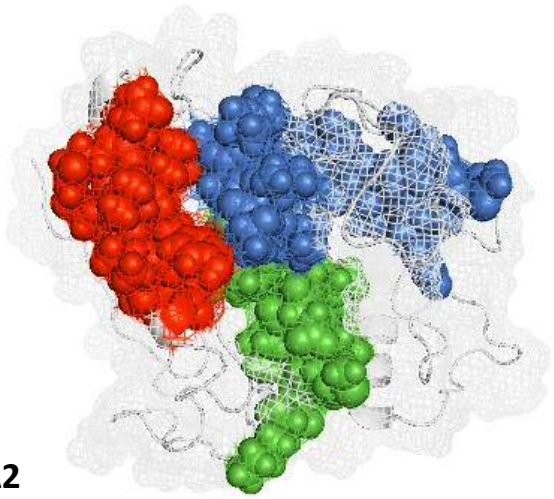
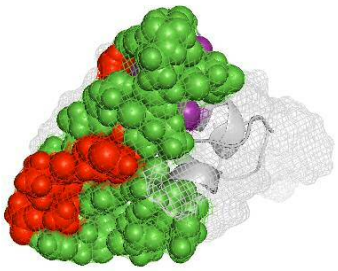
061 EKW
```

Figure 4B.



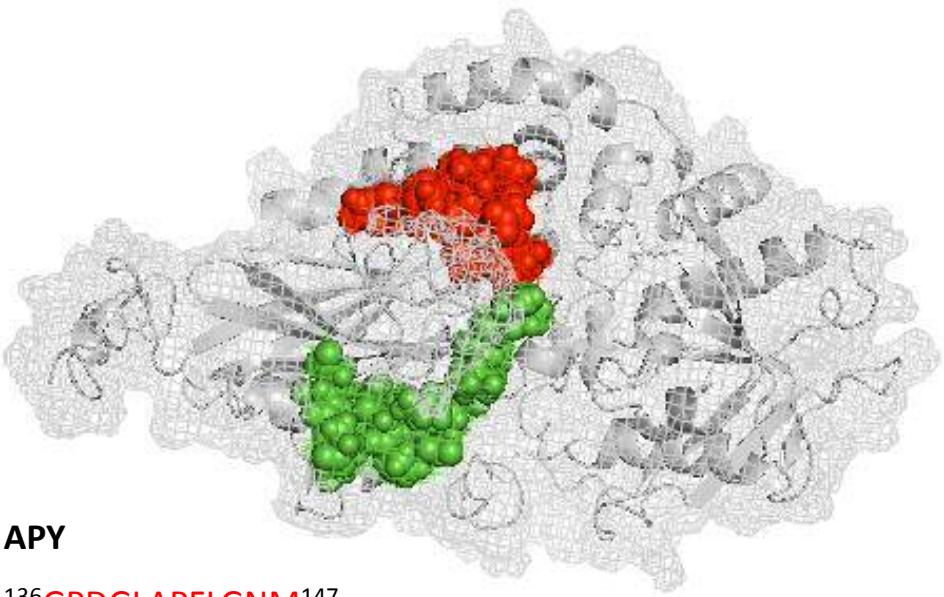
PLA2

^{4}NQTLVEQYLKCEDL^{15}
^{25}KMMSLLDRFGSA^{36}
^{37}VTLATGNTFATQNQIDAADEYVRN^{60}



MOU

^{25}ECNETPMYRCPG^{36}
^{34}CPGDEDRISGWTYDHSGSEND^{54}



APY

^{136}GPDGLAPFLGNM^{147}
^{202}GNALIKDVIPSLQEE^{216}

Figure 5.

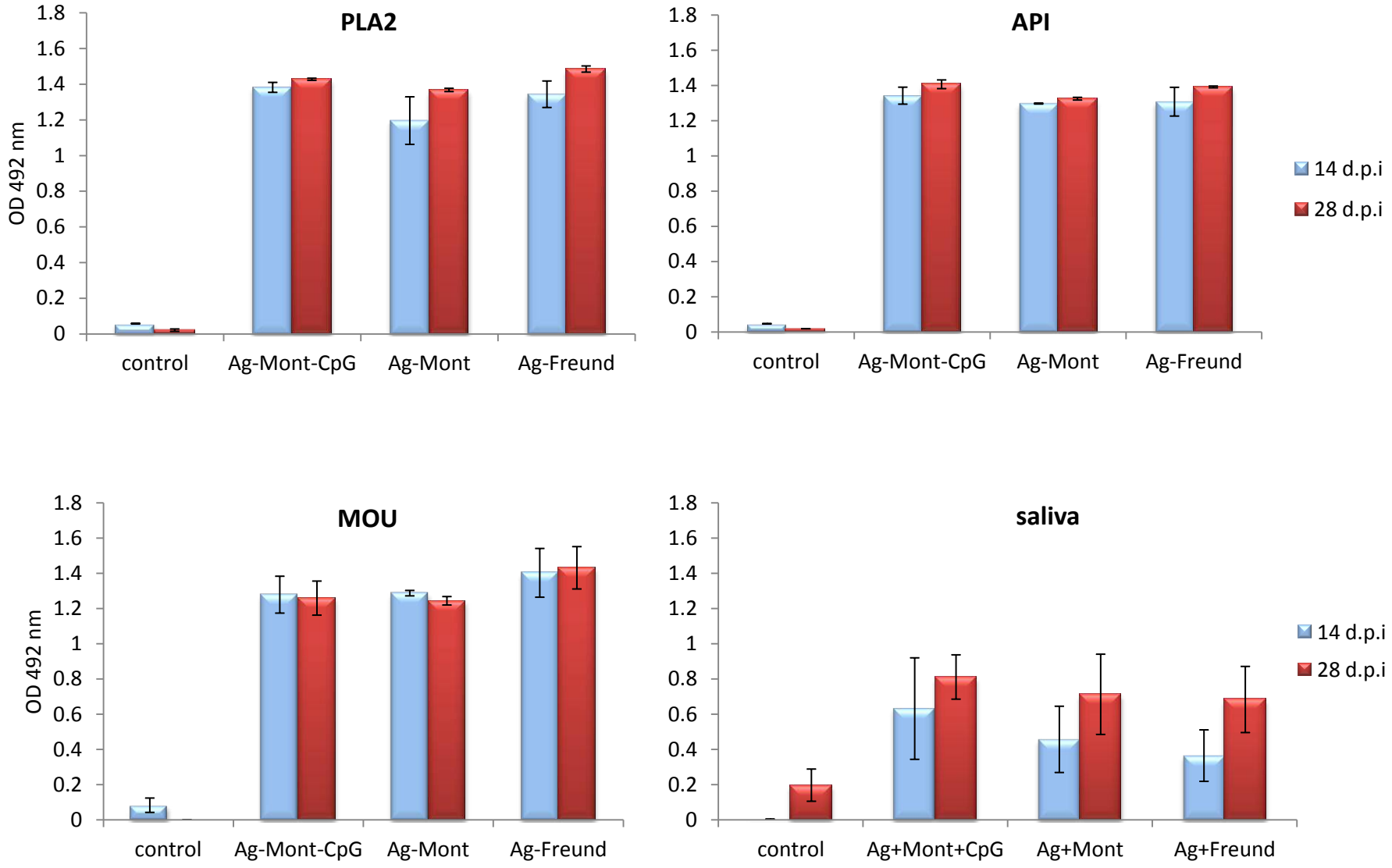
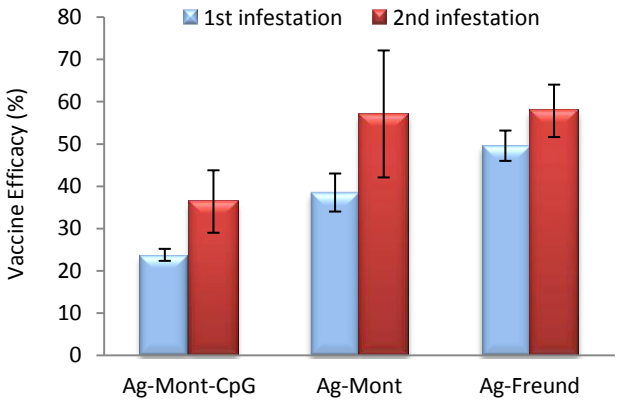

PLA2
API
MOU
saliva
OD 492 nm
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
control
Ag-Mont-CpG
Ag-Mont
Ag-Freund
Ag+Mont+CpG
Ag+Mont
Ag+Freund
14 d.p.i
28 d.p.i

Figure 6.


1st infestation
2nd infestation
Vaccine Efficacy (%)
80
70
60
50
40
30
20
10
0
Ag-Mont-CpG
Ag-Mont
Ag-Freund

Supplementary Figure 1

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Supplementary Figure 2

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Supplementary Table 1

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