

Conservation and immunogenicity of the mosquito ortholog of the tick-protective antigen, subolesin

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Abstract The control of arthropod vectors of pathogens that affect human and animal health is important for the eradication of vector-borne diseases. The ortholog of the tick-protective antigen, subolesin, was identified in *Aedes albopictus* and found to have conserved epitopes in ticks and mosquitoes. RNA interference with the tick and mosquito double-stranded RNA in three tick species resulted in significant gene knockdown and decreased tick weight and/or survival. Feeding *Anopheles atroparvus*, *Aedes caspius*, and *Culex pipiens* female mosquitoes on an *A. albopictus* subolesin hyperimmune serum resulted in $11\pm 5\%$ to $29\pm 6\%$ survival inhibition when compared to controls fed on preimmune serum. Feeding sand flies, *Phlebotomus perniciosus*, on antimosquito subolesin ortholog protein antibodies inhibited female survival and the number of larvae and adults obtained after hatching by $28\pm 22\%$ and $16\pm 3\%$, respectively, when compared to controls. Vaccination with tick and mosquito subolesin ortholog

proteins significantly reduced *Ixodes scapularis* tick infestation and weight in a similar way. However, vaccination with the recombinant mosquito subolesin ortholog antigen did not protect against *Amblyomma americanum* and *Rhipicephalus sanguineus* tick infestations. Collectively, these preliminary results provided the first evidence that development of vaccines may be possible for control of multiple arthropod vectors using subolesin orthologs but suggested that multiple antigens may be required to produce an effective vaccine.

Introduction

Diseases caused by vector-borne pathogens greatly impact human and animal health, accounting for over 20% of all emerging infectious diseases recorded between 1940 and 2004 (Jones et al. 2008). Mosquitoes are considered the

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most important vector of human diseases worldwide. *Aedes* spp. (Diptera: Culicidae) vector many viruses that cause human disease, including the exposure of over 2.5 billion people in tropical and subtropical countries to dengue fever virus (Halstead 2007). Ticks (Acari: Ixodidae) are considered to be second worldwide to mosquitoes as vectors of human diseases pathogens and the most important vector of pathogens causing cattle diseases (Peter et al. 2005; de la Fuente et al. 2008a). Phlebotomine sand flies (Diptera: Psychodidae) are also vectors of several pathogens including *Leishmania* spp. which causes various forms of human leishmaniasis (Chappuis et al. 2007).

With the exception of a few diseases such as yellow fever, vaccines against vector-transmitted diseases have not been successfully developed nor implemented, and intense use of insecticides and/or chemotherapy have resulted in an increasing number of insecticide-resistant vectors and drug-resistant pathogens (de la Fuente and Kocan 2003; Kocan et al. 2003; Willadsen 2004; Kishore et al. 2006; Sperança and Capurro 2007).

Recent developments, including transgenic vectors (Christophides 2005; Sperança and Capurro 2007), RNA interference (RNAi; Blair et al. 2006; Brown and Catteruccia 2006; de la Fuente et al. 2006a), and pathogen-derived antigens for development of transmission-blocking vaccines (Kocan et al. 2003; Kedzierski et al. 2006; Saul 2007), have shown to be a promising alternative for the control of ticks and tick-borne pathogens. However, a limited number of vaccines against arthropods resulting in generation of antigen-specific antibodies and injury to the arthropod during feeding have been reported (de la Fuente et al. 1998, 2007a; Valenzuela et al. 2001; Lal et al. 2001; Almeida and Billingsley 2002; Suneja et al. 2003; de la Fuente and Kocan 2003; Willadsen 2004; Milleron et al. 2004; Titus et al. 2006).

Subolesin is a tick-protective antigen that was recently discovered in *Ixodes scapularis* and shown by both RNAi and immunization trials using the recombinant protein to protect hosts against tick infestations, reduce tick survival and reproduction, cause degeneration of gut, salivary gland, reproductive tissues, and embryos, and reduce tick *Anaplasma marginale* and *Anaplasma phagocytophilum* infection levels (Almazán et al. 2005a, b; de la Fuente et al. 2006a, b, c; Nijhof et al. 2007; Kocan et al. 2007). Tick subolesin was shown to be an intracellular protein evolutionary conserved in invertebrate and vertebrate organisms (de la Fuente et al. 2006b; Galindo et al. 2009). Recent results suggest that subolesin is involved in regulation of gene expression, modulation of feeding, and tick reproduction and development (de la Fuente et al. 2006a, 2008b; Galindo et al. 2009).

The success of antitick vaccines in order to reduce tick infestations and interfere with development and transmis-

sion of some tick-borne pathogens (de la Fuente et al. 2006a, 2007a) and preliminary results obtained in other vector species (Valenzuela et al. 2001; Lal et al. 2001; Almeida and Billingsley 2002; Suneja et al. 2003; Milleron et al. 2004; Titus et al. 2006) have provided evidence that conserved protective antigens, such as subolesin, may be used for development of a universal vaccine with the dual target control of both arthropod infestations and reduction of vector capacity to transmit pathogens that impact human and animal health.

In this study, the ortholog of the tick-protective antigen, subolesin, was identified in mosquitoes and its protective capacity against mosquitoes, ticks, and sand flies was preliminary evaluated by RNAi and/or immunological methods. The results of these experiments provided the first evidence that development of vaccines may be possible for control of multiple arthropod vectors but suggested that multiple antigens may be required to produce an effective vaccine.

Materials and methods

Mosquitoes, ticks, and sand flies Larvae of the Asian tiger mosquito, *Aedes albopictus* Skuse, were field-collected in Sicily, Italy, and classified at the Centro Nazionale di Referenza per *Anaplasma*, *Babesia*, *Rickettsia* e *Theileria*, Istituto Zooprofilattico Sperimentale della Sicilia (IZS), Sicily, Italy. Larvae of *Anopheles atroparvus*, *Aedes caspius*, and *Culex pipiens* mosquitoes were field-collected in Spain and reared in the laboratory of JL (Departamento de Patología Animal, Facultad de Veterinaria, Zaragoza, Spain) to select adult females for in vitro feeding experiments. Mosquito were reared in mosquito breeders (BIOQUIP, Rancho Domínguez, CA, USA) at room temperature (25±2°C) and natural photoperiod.

Adult ticks (*I. scapularis*, *Amblyomma americanum*, and *Dermacentor variabilis*) were obtained from the Oklahoma State University (OSU) Tick-Rearing Facility. Larvae and nymphs were fed on rabbits and adult ticks were fed on sheep. *Rhipicephalus sanguineus* were obtained from a colony maintained at the Ixodology Laboratory from the Federal University of Uberlândia (UFU), Minas Gerais, Brazil. Ticks for this colony originated from Jaboticabal, São Paulo, Brazil, and were reared by feeding the various stages on tick-bite-naive rabbits. Animals used for tick feeding were housed at the Tick-Rearing Laboratory (OSU) or at the Ixodology Laboratory (UFU) with the approval and supervision of the Institutional Animal Care and Use Committees. Off-host ticks were maintained in a 12-h light–12-h dark photoperiod at 22–27°C and 85–95% relative humidity.

Sand flies (*Phlebotomus perniciosus*) used in this study were from an autochthonous colony established in the

laboratory of RM (Instituto de Salud Carlos III, Madrid, Spain). Flies were reared in an environmental cabinet at 28°C, 95–100% relative humidity, and a 17-h light–7-h dark photoperiod.

Cloning and sequence analysis of mosquito ortholog of tick subolesin Total RNA was extracted from *A. albopictus* larvae using TriReagent (Sigma-Aldrich, St. Louis, MO, USA) according to manufacturer's instructions. Subolesin ortholog sequences were identified by BLAST analysis of the National Center for Biotechnology Information (NCBI) non-redundant (<http://www.ncbi.nlm.nih.gov/>) and Vector Base mosquito (<http://www.vectorbase.org/>) databases in *Drosophila melanogaster* (GenBank accession number AAF50569), *Anopheles gambiae* (EAA04195), and *Aedes aegypti* (AAEL012181). With these sequences, degenerate oligonucleotide primers (AG4D855: 5'-A TGGCG/CTGC/TGCAACG/CT/CTA/GA and AG4D833: 5'-TTAG/CGAG/CAGGTAA/GCTT/AGG) were synthesized to amplify the *A. albopictus* subolesin ortholog using the Access reverse transcription polymerase chain reaction (RT-PCR) system (Promega, Madison, WI, USA) and a 52°C annealing temperature. Control reactions were performed using the same procedures but without RNA added to control contamination of the PCR. PCR products were electrophoresed on 1% agarose gels to check the size of amplified fragments by comparison to a DNA molecular weight marker (1-kb DNA ladder, Promega). Amplified fragments were resin-purified (Wizard, Promega) and cloned into the pGEM-T vector (Promega) for sequencing both strands by double-stranded dye-termination cycle sequencing (Secugen SL, Madrid, Spain). Multiple sequence alignment was performed using the program AlignX (Vector NTI Suite V 5.5, InforMax, North Bethesda, MD, USA) with an engine based on the ClustalW algorithm (Thompson et al. 1994). Antigenic peptides (≥ 8 residues) were predicted using the method of Kolaskar and Tongaonkar (1990), with a reported accuracy of about 75% (<http://immunax.dfci.harvard.edu/Tools/antigenic.pl>). Potential N- and O-glycosylation sites were predicted with NetNGlyc 1.0 (<http://www.cbs.dtu.dk/services/NetNGlyc/>) and NetOGlyc 3.0 (<http://www.cbs.dtu.dk/services/NetOGlyc/>) servers, respectively.

Expression and purification of recombinant mosquito subolesin ortholog For expression of the recombinant mosquito subolesin ortholog in *Pichia pastoris*, *A. albopictus* RNA was used to amplify the subolesin ortholog coding sequence by RT-PCR (Access RT-PCR system, Promega), using primers CZAM4D5: 5'-A CTC GAG AAA AGA GAG ATG GCG TGC GCA ACG TTG AAA C and CZAM4D3: 5'-A TCT AGA TTA GGA GAG GTA GCT AGG TGC C that introduced *Xho*I and *Xba*I

restriction sites for cloning into the expression vector pPICZ α A (Invitrogen, Carlsbad, CA, USA). In this way, mosquito subolesin ortholog was cloned under the control of the alcohol oxidase (*AOX1*) promoter, in frame with the yeast alpha-factor secretion signal but without the C-terminal c-myc/His tag due to a translation termination site introduced by CZAM4D3 primer during PCR (Canales et al. 2008). The expression constructs were sequenced and pPM4D8-1 was selected for transformation of *P. pastoris* strain X33 (Invitrogen) by electroporation as described (Invitrogen user's manuals K1710-01 and K1750-01; <http://www.invitrogen.com>).

The fermentation process was made using the X33M84D8-2 recombinant *P. pastoris* strain and the media and solutions described by Canales et al. (2008). Preinoculums and inoculums for bioreactor culture were grown in a shaker at 30°C and 250 rpm. Propagation steps were performed from long-term stock vials in 1-, 5-, and 250-ml YP medium for 12 to 24 h to reach an OD_{600 nm} between 15 and 20. Cells were finally seeded into a 5-l working volume Biostat B bioreactor (B. Braun Biotech, Melsungen, Germany) containing 3.5 l of PM medium with 40 g/l glycerol (Canales et al. 2008). A three-phase cultivation protocol was used in the fermentation process, including a batch stage followed by a fed-batch stage in glycerol and a fed-batch stage in methanol according to the *P. pastoris* Fermentation Process Guideline (Invitrogen user's manuals K1710-01 and K1750-01; <http://www.invitrogen.com>). The cells from the 5-l fermentor culture were separated by centrifugation at 3,900 \times g for 15 min. The supernatant was collected and filtered through a tandem filtration system with a 20- μ m (Sartorius AG, Göttingen, Germany), 5-, and 0.45–0.22- μ m cartridges (Millipore, Billerica, MA, USA). To purify the recombinant *A. albopictus* subolesin ortholog protein, most contaminants were first retained by size exclusion using a Sartoco[®] Slice 200 ultrafiltration system having a Hydro-sart membrane with a molecular weight cutoff of 50 kDa (Sartorius). Finally, the recombinant protein was retained, concentrated, and dialyzed against four volumes of phosphate buffer pH 8.3 using a centrifugal concentrator VIVACELL 100 with a 30-kDa cutoff (Sartorius) at 3,900 \times g.

Recombinant protein characterization by SDS-PAGE, Western blot, and matrix-assisted laser desorption/ionization time of flight mass spectrometry analyses Protein samples were analyzed by denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with a 12% polyacrylamide gel (PAGE-gel Inc., San Diego, CA, USA) under reducing conditions. Protein bands were visualized by Coomassie Brilliant Blue R250 staining. Samples were treated with dithiothreitol reducer (PAGE-gel Inc.), heated in boiling water for 5 min before loading

onto the gel, and electrophoresed for 80 min at 90-mA constant current.

Electrophoretic transfer of proteins from gels to nitrocellulose membranes (PROTRAN BA85; Schleicher and Schuell, Dassel, Germany) for Western blot analysis was done using a Minie-Genie Electrobloetter semi-dry transfer unit according to manufacturer's protocol (Idea Scientific, Corvallis, OR, USA). The supernatant of the GS115/albumin strain (Invitrogen) grown under the same conditions was used as a negative control. Western blot membranes were blocked with 5% skim milk for 1 h at room temperature, washed three times in Tris-buffered saline (TBS; 25 mmol/L Tris-HCl, 150 mmol/L NaCl, pH 7.6), and probed with sera from rabbits immunized with *I. scapularis* subolesin (1:5,000 dilution; Almazán et al. 2005a). The antiserum was diluted in 3% bovine serum albumin in TBS. Membranes were then washed three times with TBS and incubated with an antirabbit immunoglobulin G (IgG) horseradish peroxidase (HRP) conjugate (Sigma-Aldrich) diluted 1:1,000 in TBS. After washing the membranes again, color was developed using 3,3',5,5'-tetramethylbenzidine (TMB)-stabilized substrate for HRP (Promega).

The protein band corresponding to the recombinant *A. albopictus* subolesin ortholog was excised from the Coomassie-Brilliant-Blue-stained SDS polyacrylamide gel, digested with trypsin and analyzed by matrix-assisted laser desorption/ionization time of flight mass spectrometry analysis (MALDI-TOF MS) using a MALDI-TOF/TOF mass spectrometer (ABI-4700, Applied Biosystems, Inc., Foster City, CA, USA). The mass spectrum was obtained and peptide mass fingerprinting was performed for sequence identification using the MASCOT search engine (<http://matrixscience.com>) against NCBI non-redundant sequence database (<http://www.ncbi.nlm.nih.gov/>) and Mass Spectrometry Protein Sequence Database (<http://csc-fserve.hh.med.ic.ac.uk/msdb.html>) (fixed modification: carbamidomethyl (C), variable modification: oxidation (M), two missed cleavages, peptide tolerance 100 ppm).

RNA interference in ticks Homologous species-specific tick subolesin double-stranded RNAs (dsRNAs) were synthesized as described previously (de la Fuente et al. 2006b). Oligonucleotide primers AalboT75:5'-TAATACGACTCACTATAGGGTACTATGGCGTGCGCAACGTTGAAA-3' and AalboT73:5'-TAATACGACTCACTATAGGGTACTTTAGGAGAGGTAGCTAGGTGC-3' homologous to *A. albopictus* subolesin ortholog containing T7 promoters were used for in vitro transcription and synthesis of dsRNA as described previously (de la Fuente et al. 2006b), using the Access RT-PCR system (Promega) and the Megascript RNAi kit (Ambion, Austin, TX, USA). The dsRNA was purified and quantified by spectrometry.

Unfed *I. scapularis*, *A. americanum*, and *D. variabilis* female ticks (30 ticks per group) were injected with approximately 0.5 μl (5×10^{10} – 5×10^{11} molecules per microliter) dsRNA in the lower-right quadrant of the ventral surface of the exoskeleton of ticks (de la Fuente et al. 2006b). The injections were done using a 10- μl syringe with a 1-in. 33-gauge needle (Hamilton, Bonaduz, Switzerland). Control ticks were injected with homologous tick subolesin dsRNA (positive control; $N=30$) or injection buffer (10 mM Tris-HCl, pH 7, 1 mM ethylenediaminetetraacetic acid) alone (saline negative control; $N=10$; de la Fuente et al. 2006b). Ticks were kept in a humidity chamber for 1 day after which they were allowed to feed with 20 males per group on a sheep in individual stockinettes for 10 days. Unattached ticks were removed 48 h after tick infestation. Ticks completely fed or removed after 10 days were counted and weighted. Tick mortality was evaluated as the ratio of dead ticks after feeding on the sheep to the total number of fed ticks and compared between dsRNA-injected and controls by χ^2 test as implemented in Mstat 4.01 ($\alpha=0.01$). Tick weight was compared between dsRNA-injected and controls by Student's *t* test with unequal variance ($P=0.05$). Internal organs were dissected and stored in RNALater (Ambion) for RNA extraction and determination of the subolesin messenger RNA (mRNA) levels by real-time RT-PCR.

Real-time RT-PCR analysis of subolesin mRNA levels Total RNA was extracted from five individual ticks from each group using TriReagent (Sigma) according to manufacturer's instructions. Subolesin mRNA levels were determined as described previously (de la Fuente et al. 2006b). Real-time RT-PCR was performed using the QuantiTect SYBR Green RT-PCR kit (Qiagen, Valencia, CA, USA) and a Bio-Rad iQ5 thermal cycler (Hercules, CA, USA) following manufacturer's recommendations. Amplification efficiencies were normalized against tick β -actin using the comparative Ct method (de la Fuente et al. 2007b). Subolesin mRNA levels were compared between dsRNA-injected and control ticks by Student's *t* test with unequal variance ($P=0.05$) and average mRNA levels were used to calculate percent silencing in dsRNA-injected ticks with respect to controls.

Characterization of the protective capacity of recombinant mosquito subolesin ortholog protein Two sets of experiments were used to characterize the protective capacity of the recombinant mosquito subolesin ortholog protein. In the first set of experiments, the effect of antimosquito subolesin ortholog protein antibodies was evaluated on homologous (mosquitoes) and heterologous (sand fly) insect vector species. In the second set of experiments, the recombinant mosquito antigen was used in vaccination trials to evaluate

its efficacy for the control of *I. scapularis*, *A. americanum*, and *R. sanguineus* tick infestations.

Effect of antibodies against mosquito subolesin ortholog protein on mosquitoes and sand flies Immunization of rabbits with the recombinant mosquito subolesin ortholog protein was conducted for a preliminary evaluation of the effect of antibodies against mosquito subolesin ortholog protein on mosquito (*A. atroparvus*, *A. caspius*, and *C. pipiens*) and sand fly (*P. perniciosus*) fecundity and/or survivorship. Two New Zealand White rabbits were each immunized three times at weeks 0, 4, and 8 with 1-ml doses containing 50 µg of purified recombinant mosquito subolesin ortholog protein formulated as described previously (Canales et al. 2008). Rabbits were injected subcutaneously using a 1-ml tuberculin syringe and a 27.5-G needle. Two weeks after the last immunization, blood samples were collected from each rabbit and the serum was then separated by centrifugation and stored at -20°C. Preimmune serum was obtained from each rabbit before immunization to serve as negative control samples. Rabbits were cared for in accordance with standards specified in the Guide for Care and Use of Laboratory Animals. Serum antibodies to the recombinant mosquito subolesin ortholog protein were confirmed by Western blot as described before and were used to artificially feed female mosquitoes and sand flies to evaluate arthropod fecundity and/or survivorship as described previously (Molina 1991a, b; Molina et al. 1999; Munstermann 1997).

Fifteen female mosquitoes were used in each experiment. Mosquitoes were fed for 2–3 h on cotton embedded in a solution containing 10% sugar and 90% mosquito subolesin ortholog protein hyperimmune serum or preimmune serum for controls (Munstermann 1997). Fed mosquitoes showed enlarged abdomens when compared to unfed individuals. Mosquito mortality was evaluated 24–48 h after feeding. Data were analyzed statistically to compare results of mosquitoes fed on immune and control sera by χ^2 test when the number of experiments was (exp. no.) ≥ 2 ($\alpha=0.01$) and by Student's *t* test for exp. no. >2 ($P=0.01$).

Two groups of 175 female sand flies each were artificially fed on preimmune control or recombinant mosquito subolesin ortholog protein hyperimmune sera. Fifty fed females per group were transferred to individual flasks for analysis of oviposition, eclosion, and development of adult flies. Two independent experiments were conducted to evaluate the number of oviposited females and the number of larvae and adults produced on each experiment. Data were analyzed statistically to compare results of sand flies fed on immune and control sera by χ^2 test as implemented in Mstat 4.01 ($\alpha=0.01$).

Immunization trial against *I. scapularis* and *A. americanum* tick infestations The immunization trial in sheep

against *I. scapularis* and *A. americanum* infestations was designed to compare the efficacy of the recombinant mosquito subolesin ortholog and *I. scapularis* subolesin antigens on adult *I. scapularis* and *A. americanum* infestations. For this trial, six sheep were randomized into three groups of two sheep each. Two injections were administered subcutaneously using a 6-ml syringe and an 18.5-G needle 1 month apart with 1-ml doses that contained 100-µg recombinant mosquito subolesin ortholog protein, *I. scapularis* subolesin antigen (Almazán et al. 2005a), or saline adjuvated in Montanide ISA 50 V2 (Seppic, Paris, France). The sheep were cared for in accordance with standards specified in the OSU Guide for Care and Use of Laboratory Animals. Two weeks after the second immunization, sheep were infested with 50 adult pairs of *I. scapularis* and *A. americanum* per sheep in individual cotton stockinette cells glued to the side of the animal. After 48 h, all unattached ticks were removed and counted. Engorged female ticks were collected daily from each sheep for up to 10 days and then counted and weighed. The number of ticks attached per animal (RLi), the ratio of the number of replete ticks (RLn) to the ticks attached per sheep (RLn/RLi), and the tick weight were compared between immunized and control sheep using the Student's *t* test ($P=0.05$). The inhibition of tick infestations (*I*) and the reduction of tick weight (TW) were calculated for each immunized group with respect to the saline-immunized controls as described previously (Almazán et al. 2005a). Immunizations and tick collections were done blinded and the key to the experimental groups was not opened until the end of the experiment.

Immunization trial against *R. sanguineus* infestations The immunization trial against *R. sanguineus* infestations on dogs was designed to test the efficacy of recombinant mosquito subolesin ortholog as a vaccine antigen against this tick species. Twelve 5- to 6-month-old mongrel ($N=7$) and red heeler ($N=5$) male ($N=8$) and female ($N=4$) dogs were used in the experiment.

Prior to the immunizations, all the dogs were vaccinated against canine distemper and hepatitis, parvovirus, leptospirosis, and rabies and treated for intestinal helminthes. The dogs were fed commercial ration and water was provided “ad libitum” throughout the trial. All dogs had previously been exposed to natural *R. sanguineus* infestations. The dogs were housed in a separate section of the Veterinary Hospital of the Federal University of Uberlândia, Minas Gerais, Brazil according to the Colégio Brasileiro de Experimentação Animal and donated at the end of experiment.

For the immunization trial, the dogs were randomly allocated into two groups of six animals each. Each experimental group had two females and either three or

four mongrel dogs. The dogs were immunized twice, 1 month apart with 1-ml doses containing 100 µg of the recombinant antigen or saline adjuvated in Montanide ISA 50 V2 (Seppic, Paris, France). Thirty days after the last immunization, dogs were infested with 25 female and 30 male ticks, 50 nymphs, and 250 larvae, with each tick stage being placed in individual chambers glued to the shaved backs of the animals as described previously (Szabó et al. 1995). Chambers were monitored daily during the 10-day feeding period. Engorged and detached ticks were collected, placed in plastic vials, and held in humidity chambers. Adult female ticks were placed in individual vials and nymphs and larvae were collected daily and stored in batches. The tick yield ($RL_n/RL_i \times 100$), tick weight, egg mass weight, egg hatching rate, and molting were calculated and compared between ticks fed on vaccinated and control dogs using Student's *t* test ($P=0.05$). The inhibition of tick infestations (*I*), oviposition (*O*), egg fertility (*F*), and molting (*M*) and the reduction of tick weight (TW) were calculated for ticks fed on the immunized animals as compared with those fed on the control dogs as described previously (Canales et al. 1997; Almazán et al. 2005a, b). Immunizations and tick collections were done blinded and the key to the experimental groups was not opened until the end of the experiment.

Characterization of the immune inflammatory response in dogs immunized with the recombinant mosquito subolesin ortholog protein The inflammatory response of dogs to tick infestations was studied using a cutaneous hypersensitivity test after immunization with the recombinant mosquito subolesin ortholog protein. The tick antigen extract was prepared from unfed adult *R. sanguineus* as described previously (Szabó et al. 1995). Dogs were inoculated 45 days after last immunization with 25-µg tick extract in 0.1-ml phosphate-buffered saline (PBS) into the dermis of the left ear. Dogs were inoculated in a similar manner into the right ear with PBS to serve as controls for detection of non-specific inflammation. Inflammatory reactions were evaluated by measuring skin thickness at 10 min and 1, 6, 24, 48, 72, and 96 h post-injection (hpi). The thickness of injection sites was measured in both ears three times at each interval with a thickness gage (Mitutoyo, Kawasaki, Japan) and results were expressed as mean percent changes in the ear thickness with respect to preinjection values. The final increase in ear thickness induced by unfed adult tick extract was given by subtracting values measured in the right ear from those of the left ear and compared between immunized and control dogs by unpaired Student's *t* test ($P=0.05$).

Characterization of the immune response in immunized animals by ELISA The antibody response against recombinant proteins in immunized and control sheep was

evaluated using an antigen-specific indirect enzyme-linked immunosorbent assay (ELISA). Antibody titers were determined in sera collected before each immunization and 2 weeks after the last immunization. Purified recombinant proteins (0.1 µg per well) were used to coat ELISA plates overnight at 4°C. Sera were serially diluted to 1:10, 1:100, and 1:1,000 in PBS with Tween (PBST; PBS/0.5% Tween 20, pH 7.2) and 10% fetal bovine serum (Sigma). The plates were incubated with the diluted sera for 1 h at 37°C and then incubated with 1:10,000 rabbit antisheep IgG-HRP conjugates (Sigma) for 1 h at 37°C. The color reaction was developed with 3,3',5,5'-tetramethylbenzidine (Sigma) and the $OD_{450\text{ nm}}$ was determined. After incubation, the plates were washed with PBST. Antibody titers were considered positive when they yielded an $OD_{450\text{ nm}}$ value at least twice as high as the negative control preimmune serum. Antibody titers in immunized sheep were expressed as the inverse of the serum dilution at which a positive $OD_{450\text{ nm}}$ ($OD_{\text{test sheep}} - OD_{\text{control}}$) was obtained.

Antibody responses against recombinant mosquito subolesin ortholog protein was evaluated in immunized and control dogs with sera collected 2 weeks after the last immunization by antigen-specific indirect ELISA as described above with the preimmune sera serving as controls, with the exception that ELISA plates were coated with 0.5 µg per well of the purified recombinant protein; sera were diluted to 1:800 in PBST and 5% skim milk and a goat antidog IgG-HRP conjugate (1:12,500; Sigma) was used. Sera collected from two control and one immunized dogs were lost during the experiment and therefore were not included in the ELISA.

Sequence accession number The GenBank accession number for *A. albopictus* subolesin ortholog sequence is EU637024.

Results

Cloning and sequence analysis of the A. albopictus ortholog for tick subolesin

The sequence coding for the *A. albopictus* subolesin ortholog was cloned by RT-PCR and compared with reported mosquito and tick sequences. *A. albopictus* subolesin ortholog encoded for a predicted protein of 200 amino acids and a molecular weight of 22.5 kDa (Fig. 1). Pairwise nucleotide and amino acid sequence alignments were conducted between mosquito and tick subolesin ortholog sequences (Table 1). Mosquito subolesin ortholog sequences were 76–89% identical and 84–95% similar at the nucleotide and amino acid levels, respectively (Table 1).

Table 1 Nucleotide (above diagonal) and amino acid (below diagonal) identity/similarity for all pairwise comparisons of mosquito and tick subolesin ortholog sequences

	<i>A. gambiae</i>	<i>A. albopictus</i>	<i>A. aegypti</i>	<i>A. americanum</i>	<i>D. variabilis</i>	<i>I. scapularis</i>	<i>R. sanguineus</i>
<i>A. gambiae</i>	100	80	76	46	46	47	45
<i>A. albopictus</i>	84	100	89	51	50	51	49
<i>A. aegypti</i>	84	95	100	49	49	50	49
<i>A. americanum</i>	42	41	42	100	76	73	74
<i>D. variabilis</i>	43	44	45	83	100	76	90
<i>I. scapularis</i>	44	44	45	78	83	100	73
<i>R. sanguineus</i>	42	44	44	79	92	80	100

Sequences were aligned and percent identity/similarity was determined using the program AlignX (Vector NTI Suite V 5.5, InforMax, North Bethesda, MD, USA)

scapularis subolesin antibodies with an estimated molecular weight of 36 kDa (Fig. 2). The *A. albopictus* subolesin ortholog protein had one potential N-glycosylation and 15 potential O-glycosylation sites (data not shown). Therefore, the greater-than-predicted molecular weight of the recombinant *A. albopictus* subolesin ortholog protein was most likely due to glycosylation of recombinant protein by *P. pastoris*, as demonstrated by the wide appearance of the protein band in the SDS-PAGE and Western blot (Fig. 2). The protein band corresponding to the recombinant *A. albopictus* subolesin ortholog was analyzed by MALDI-

TOF MS and corroborated the predicted protein sequence (data not shown). After purification, the mosquito recombinant protein was used in immunization trials in rabbits, sheep, or dogs to determine its protective capacity against mosquitoes, sand flies, and ticks.

Effect of RNAi with mosquito subolesin ortholog sequence in ticks

A preliminary experiment was conducted using *A. albopictus* subolesin ortholog dsRNA to analyze subolesin mRNA levels in ticks after RNAi. Tick species-specific subolesin dsRNA was used as positive control. As validated in previous experiments (de la Fuente et al. 2006b), the injection buffer alone was used as negative control. Additionally, injection in *I. scapularis* of the unrelated Rs86 dsRNA as negative control gave results similar to those described in Table 2 (data not shown). The expression of tick subolesin was silenced after RNAi with both tick and mosquito dsRNA (Table 2). However, except for *A. americanum*, silencing of subolesin expression was more pronounced with tick (80–100%) than with mosquito (40–57%) dsRNA. As demonstrated previously, subolesin RNAi using the tick subolesin dsRNA decreased tick weight and increased tick mortality in all three tick species analyzed (Table 2). However, RNAi with mosquito dsRNA produced variable results. Tick weight was significantly decreased in *D. variabilis* and *I. scapularis*, while tick mortality increased only in *A. americanum* (Table 2).

Characterization of the protective capacity of anti-recombinant mosquito subolesin ortholog protein antibodies in mosquitoes and sand flies

The effect of antibodies against the recombinant mosquito subolesin ortholog protein on mosquito mortality was evaluated in three mosquito species (Table 3). The results suggested that feeding mosquitoes on immune serum

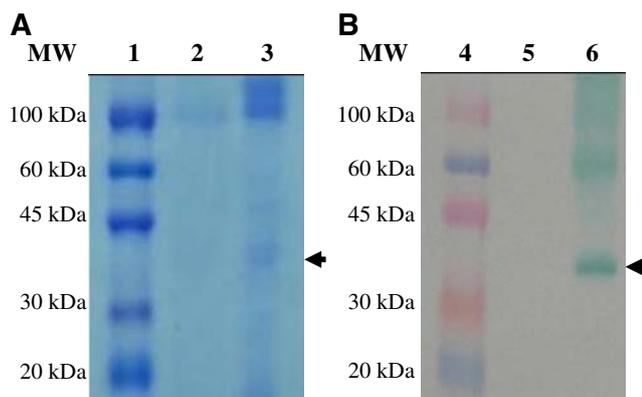


Fig. 2 Secretion of recombinant mosquito subolesin ortholog protein by *P. pastoris*. Coomassie-stained SDS-PAGE (a) and Western blot analysis (b) of the fermentation culture supernatants after 72 h growing in methanol. Samples of 15 μ l were loaded in each well. Membranes for Western blot were probed with serum from rabbits immunized with *I. scapularis* tick subolesin diluted 1:1,000. Membranes were then washed three times with TBS and incubated with an antirabbit IgG-HRP conjugate (Sigma) diluted 1:1,000 in TBS. After washing the membranes again, color was developed using TMB-stabilized substrate for HRP (Promega). Lanes 1 and 4: molecular weight markers (MW; ColorBurst, Sigma). Lanes 2 and 5: culture supernatants of the *P. pastoris* GS115/albumin-negative control strain. Lanes 3 and 6: culture supernatant of X33M84D8-2 strain expressing recombinant *A. albopictus* subolesin ortholog protein. The position of recombinant protein is indicated with arrows

Table 2 Effect of targeting subolesin expression by RNAi on tick feeding and mortality rate

Tick species	Subolesin expression silencing after RNAi (%) ^a		Tick weight (mg) ^b			Tick mortality (%) ^c		
	Tick dsRNA	Mosquito dsRNA	Tick dsRNA	Mosquito dsRNA	Control	Tick dsRNA	Mosquito dsRNA	Control
<i>A. americanum</i>	51±11*	49±2*	423±268**	669±210	688±96	20 ^d	10 ^d	0
<i>D. variabilis</i>	80±2*	57±1*	394±362**	561±249**	787±318	25 ^d	13	10
<i>I. scapularis</i>	100±0*	40±1*	66±29**	179±47**	213±27	37 ^d	13	10

* $P < 0.05$; ** $P < 0.05$;

^a Subolesin mRNA levels were determined by real-time RT-PCR after RNAi. Amplification efficiencies were normalized against tick β -actin using the comparative Ct method. mRNA levels were compared between infected and uninfected ticks by Student's t test with unequal variance ($N=5$) and average mRNA levels were used to calculate percent silencing in dsRNA-injected ticks with respect to saline-injected controls

^b Ticks that completed feeding and those removed after 10 days of feeding were weighed individually and average \pm SD was calculated and compared between dsRNA-injected and saline control ticks by Student's t test with unequal variance ($N=30$)

^c Tick mortality was evaluated as the ratio of dead ticks after feeding on the sheep to the total number of fed ticks and compared between dsRNA-injected and saline controls by χ^2 test ($N=30$)

^d $\alpha < 0.01$

reduced survival for *A. atroparvus* and *C. pipiens* when compared to controls fed on preimmune serum (Table 3).

Two experiments were conducted for the preliminary characterization of the effect of anti-*A. albopictus* subolesin ortholog protein antibodies on sand fly fecundity and survival (Table 4). Although results varied between experiments, the results suggested an effect of antibodies against mosquito subolesin ortholog protein in reducing fly survival and fecundity (Table 4).

Characterization of the protective capacity of recombinant mosquito subolesin ortholog protein on *I. scapularis* and *A. americanum* adult tick infestations in sheep

Two sheep per group were immunized with recombinant *I. scapularis* or *A. albopictus* subolesin ortholog proteins or saline to compare the effect of both antigens on *I.*

scapularis and *A. americanum* adult tick infestations. All immunized sheep developed antigen-specific antibodies after the first immunization and antibody levels remained similar or increased after the second immunization (Fig. 3a, b). The tick and mosquito subolesin ortholog proteins shared antigenic epitopes as shown above by sequence characterization (Fig. 1) and Western blot analysis of recombinant *A. albopictus* protein with anti-*I. scapularis* antibodies (Fig. 2). In agreement with these results, sera from sheep immunized with the mosquito antigen (S4 and S5) reacted with tick subolesin (Fig. 3a) and serum from sheep S9 immunized with the tick antigen recognized the recombinant *A. albopictus* subolesin ortholog protein (Fig. 3b). Antibody titers were generally higher against the homologous antigen (S9 and S2 in Fig. 3a and S4 and S5 in Fig. 3b). Control animals and preimmune sera did not react against recombinant antigens (Figs. 3a, b).

Table 3 Effect of anti-mosquito subolesin ortholog protein antibodies on mosquito mortality

Mosquito species	Mean \pm SD dead mosquitoes		Inhibition of mosquito survival (%; mean \pm SD)
	Fed on immune serum	Fed on control serum	
<i>A. atroparvus</i> ($N=15$, exp. no.=2)	4.0 \pm 2.8 ^a	3.0 \pm 2.1	29 \pm 6
<i>C. pipiens</i> ($N=60$, exp. no.=4)	2.5 \pm 1.3 ^{a,*}	1.0 \pm 0.8	11 \pm 5
<i>A. caspius</i> ($N=15$, exp. no.=1)	5	1	29

Fifteen female mosquitoes were used in each experiment. The total number of mosquitoes (N) and experiments used for analysis (exp. no.) are shown. Mosquito mortality was evaluated 24–48 h after feeding on mosquito subolesin ortholog protein immune serum or preimmune serum for controls. The inhibition of mosquito survivorship was calculated with respect to the control group. Data were analyzed statistically to compare results of mosquitoes fed on immune and control sera by χ^2 test for exp. no. ≥ 2 and by Student's t test for Exp. no. > 2

* $P < 0.01$

^a $\alpha < 0.01$

Table 4 Effect of anti-mosquito subolesin ortholog protein antibodies on sand fly, *P. perniciosus*, fecundity and survival

Biological parameter	Fed on immune serum (mean \pm SD)	Fed on control serum (mean \pm SD)	Inhibition (%; mean \pm SD)
No. of survived females that laid eggs	24 \pm 15 ^a	31 \pm 11	28 \pm 22
No. of larvae obtained	236 \pm 190 ^a	287 \pm 237	16 \pm 3
No. of adults obtained	215 \pm 171 ^a	261 \pm 215	16 \pm 3

Two independent experiments with 175 female sand flies each were conducted. The inhibition of sand fly survival and production of larvae and adults were calculated with respect to the control group. Data were analyzed statistically to compare results of sand flies fed on immune and control sera by χ^2 test

^a $\alpha < 0.01$

The results of the vaccine trial were similar for both tick and mosquito antigens (Table 5). Vaccination with either antigen protected sheep against *I. scapularis* as demonstrated by the inhibition of adult tick infestations and reduction of tick weight (Table 5). However, due to the notable animal-to-animal variation observed in the data obtained from the adult *A. americanum* infestations, neither antigen provided statistically significant differences between vaccinated and control animals (Table 5). Nonetheless, tick infestation was inhibited in a similar way for *I. scapularis* and *A. americanum* in sheep S9 and S5 immunized with tick and mosquito antigens, respectively (Table 5).

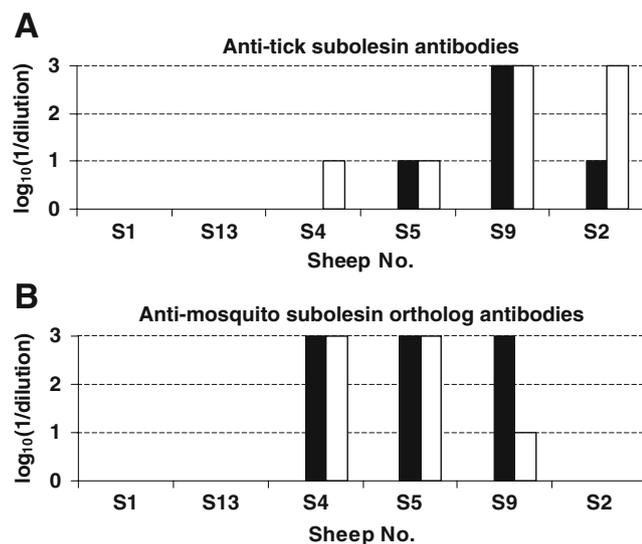


Fig. 3 Antibody response to recombinant *I. scapularis* and *A. albopictus* subolesin ortholog proteins in immunized sheep. Antibody titers were determined for (a) tick and (b) mosquito antigens by ELISA and expressed as the inverse of serum dilution at which a positive OD_{450nm} (OD_{test sheep} - OD_{control}) was obtained. Antibody titers were determined in preimmune sera (gray bars) and in sera collected before second immunization (black bars) and 2 weeks after last immunization (white bars). Sheep (S) 1 and 13 were injected with saline/adjuvant and used as controls. S4 and S5 were immunized with the recombinant mosquito subolesin ortholog protein. S9 and S2 were immunized with the recombinant tick antigen. Control animals and preimmune sera did not react against recombinant antigens

Characterization of the protective capacity of recombinant mosquito subolesin ortholog protein on *R. sanguineus* tick infestations in dogs

Dogs immunized with the recombinant *A. albopictus* subolesin ortholog protein developed antibodies specific for this protein (Fig. 4). Vaccinated animals demonstrated an immediate inflammatory response to tick extract shown by ear thickness increase 10 min after tick extract inoculation that remained high until the last measurement (Fig. 5). Control animals had a transient reaction to the tick extract, 10 min and 48 h after inoculation. Inflammatory reactions in response to tick extract injection were significantly greater at 6 and 96 hpi ($P < 0.05$) in the vaccinated dogs as compared with the controls. The animal-to-animal variation observed among dogs in the vaccination experiment affected the analysis of results. Nonetheless, a trend towards reduction of oviposition and nymphal and larval *R. sanguineus* infestations was observed (Table 6). However, the results of the vaccine trial failed to demonstrate a statistical significant effect of mosquito subolesin ortholog protein against *R. sanguineus* infestations in dogs (Table 6).

Discussion

Recent research toward the development of vaccines for the control of arthropod vectors of pathogens affecting human and animal health has been concentrated on the most important vector species of ticks, mosquitoes, and sand flies (Valenzuela et al. 2001; Lal et al. 2001; Almeida and Billingsley 2002; Suneja et al. 2003; de la Fuente and Kocan 2003; Willadsen 2004; Milleron et al. 2004; Titus et al. 2006). However, despite this research focus, the only two arthropod vaccines that were commercialized are for the control of cattle tick infestations (recently reviewed by de la Fuente et al. 2007a).

An important advantage of arthropod vaccines will likely be the ability to reduce or prevent transmission of several pathogens through immunization of reservoir hosts and

Table 5 Effect of vaccination with recombinant tick and mosquito subolesin ortholog proteins on the control of adult *I. scapularis* and *A. americanum* tick infestations

Group	Sheep no.	RLi	RLn	RLn/RLi	<i>I</i> (%)	Tick weight (mg)	TW (%)
<i>I. scapularis</i>							
Control	S1	43	40	0.93	–	231±41	–
	S13	47	45	0.96	–	248±46	–
	Mean±SD	45±3	43±4	0.95±0.02	–	240±44	–
Tick subolesin	S2	50	42	0.84	12	205±51	15
	S9	49	43	0.88	8	236±49	2
	Mean±SD	50±1	43±1	0.86±0.03*	10±3	220±54*	9±9
Mosquito subolesin ortholog	S4	47	39	0.83	13	232±57	3
	S5	50	41	0.82	14	224±51	7
	Mean±SD	49±2	40±1	0.83±0.01*	13±1	229±54*	5±3
<i>A. americanum</i>							
Control	S1	48	46	0.96	–	801±165	–
	S13	48	46	0.96	–	680±144	–
	Mean±SD	48±0	46±0	0.96±0.00	–	740±166	–
Tick subolesin	S2	46	46	1.00	–4	706±137	5
	S9	46	42	0.91	5	759±136	–2
	Mean±SD	46±0	44±3	0.96±0.06	0.5±6	738±131	2±5
Mosquito subolesin ortholog	S4	50	50	1.00	–4	746±102	–1
	S5	49	36	0.73	23	644±173	13
	Mean±SD	50±1	43±10	0.87±0.19	10±19	703±145	6±10

Groups of two sheep each were immunized with recombinant *I. scapularis* tick subolesin, *A. albopictus* mosquito subolesin ortholog, or saline/adjuvant for controls and infested with 50 adult pairs of *I. scapularis* and *A. americanum* per sheep, using separate cotton stockinette cells glued to the side of the animal. The number of ticks attached per animal (RLi), the ratio of the number of replete ticks (RLn) to the ticks attached per sheep (RLn/RLi), and the tick weight were compared between antigen-immunized and control saline-immunized sheep by Student's *t* test. The inhibition of tick infestation (*I*) and the reduction of tick weight (TW) were calculated with respect to the control group

* $P < 0.05$

human and animal populations at risk (de la Fuente and Kocan 2003). In addition, the identification of evolutionary conserved protective antigens may lead to development of a multi-target vaccine directed at the control of several vector species.

As the first step toward the goal of a multi-target arthropod vaccine, we used the tick-protective antigen, subolesin, which has been shown to be evolutionary conserved in vertebrate and invertebrate organisms including Ixodida and insects and effective as a vaccine antigen

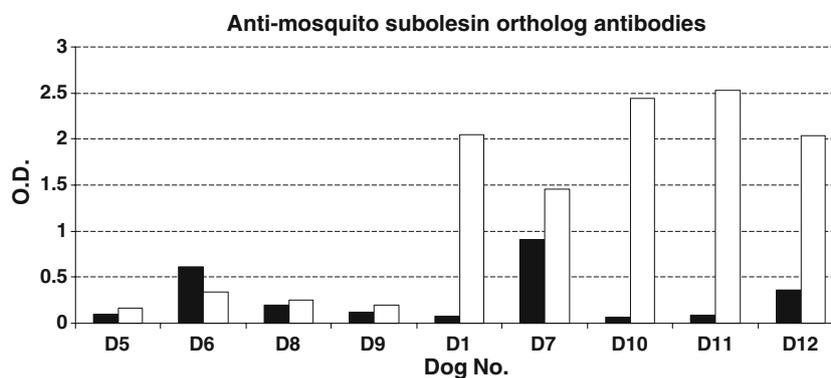


Fig. 4 Antibody response to recombinant *A. albopictus* subolesin ortholog protein in immunized dogs. Antibody titers were determined for the mosquito antigen by ELISA and expressed as absorbance OD_{450nm}. Antibody titers were determined in sera collected before first

immunization (black bars) and 2 weeks after the last immunization (white bars). Dogs (D) 5, 6, 8, and 9 were injected with saline/adjuvant and used as controls. D1, D7, D10, D11, and D12 were immunized with the recombinant mosquito subolesin ortholog protein

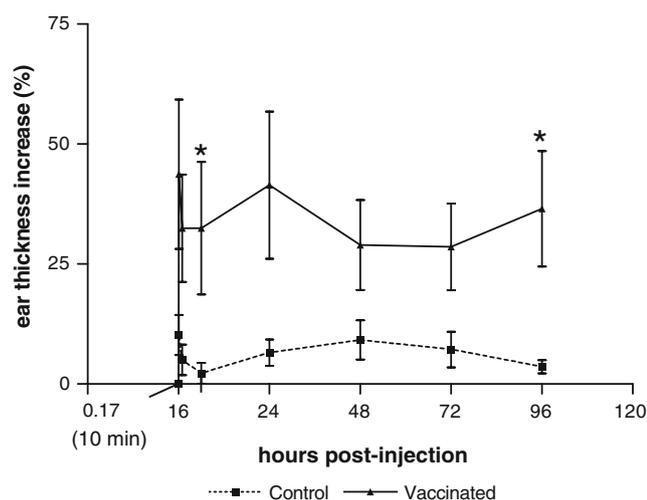


Fig. 5 Effect of vaccination on dog immune inflammatory response to ticks. The cutaneous hypersensitivity test was conducted using a tick antigen extract prepared from unfed adult *R. sanguineus* ticks. The tick extract was injected 45 days after last vaccination dose into the dermis of the left ear of dogs and PBS only was injected into the right ear as control for non-specific inflammation. Inflammatory reactions were evaluated by measuring skin thickness at 10 min and 1, 6, 24, 48, 72, and 96 h post-injection. The thickness of injection sites was measured in both ears three times at each interval and results were expressed as mean percent changes in the ear thickness with respect to preinjection values. The final increase in ear thickness induced by unfed tick extract was given by subtracting values measured in the right ear from those of the left ear and compared between immunized and control dogs by unpaired Student's *T* test (* $P < 0.05$)

for the control of tick infestations (Almazán et al. 2005a, b; de la Fuente et al. 2006b; Galindo et al. 2009). Evolutionary studies have shown that subolesin orthologs evolved from longer sequences in insects to shorter sequences in *Rhipicephalus* spp. ticks (de la Fuente et al. 2006b). Therefore, in this research, we cloned and characterized the *A. albopictus* mosquito subolesin ortholog, which is ancestral to tick subolesin and most likely encodes for a protein with common protective epitopes in mosquitoes and ticks.

Analysis of subolesin ortholog sequences in mosquitoes and ticks revealed a high degree of sequence conservation among these organisms and suggested the presence of conserved antigenic epitopes. Immune cross-reactivity between *I. scapularis* and *A. albopictus* subolesin ortholog proteins shown by Western blot and ELISA confirmed the presence of conserved epitopes in these proteins. In addition, the immune inflammatory response reaction in tick-infestation-sensitized dogs in response to inoculation with *R. sanguineus* tick extract (Szabó et al. 1995) was more intense in dogs immunized with recombinant mosquito subolesin ortholog protein than in the control dogs. This result also provided evidence of the immune cross-reactivity between mosquito and *R. sanguineus* subolesin ortholog proteins. Collectively, these results suggested that tick and mosquito subolesin ortholog proteins share antigenic epitopes that may be used to elicit a protective response in immunized hosts.

Table 6 Effect of vaccination with recombinant mosquito subolesin ortholog protein on the control of *R. sanguineus* tick infestations

Biological parameter	Vaccinated (mean ± SD)	Control (mean ± SD)	Inhibition (%)
Adult females			
Tick yield (%)	70.0±27.2	69.3±36.5	<i>I</i> =0
Tick weight (mg)	141.9±11.8	138.9±10.6	TW=0
Egg mass weight per tick (mg)	52.2±37.1	59.7±24.6	<i>O</i> =13
Egg hatching rate (%)	70.1±23.1	69.4±16.2	<i>F</i> =0
Nymphs			
Tick yield (%)	50.7±14.6	61.3±40.0	<i>I</i> =17
Tick weight (mg)	4.0±0.4	3.8±0.3	TW=0
Molting rate (%)	88.7±15.9	91.8±7.8	<i>M</i> =3
Larvae			
Tick yield (%)	23.6±14.1	40.3±30.5	<i>I</i> =41
Tick weight (mg)	0.28±0.08	0.29±0.11	TW=3
Molting rate (%)	90.7±10.5	77.0±31.6	<i>M</i> =0

Groups of six dogs each were immunized with recombinant *A. albopictus* mosquito subolesin ortholog protein or saline/adjuvant for controls. The tick yield (RLn/RLi×100), weight, egg mass weight, egg hatching rate, and molting were calculated and compared between vaccinated and control dogs by Student's *t* test ($P > 0.05$). The inhibition of tick infestation (*I*), oviposition (*O*), egg fertility (*F*), molting (*M*), and the reduction of tick weight (TW) were calculated with respect to the control group

The first evidence of the possibility of using the *A. albopictus* subolesin ortholog to affect tick physiology was obtained in RNAi experiments. RNAi with the mosquito dsRNA in three tick species resulted in decreased tick weight or survival. Regions of 23 nucleotides containing stretches of identical nucleotides may suffice for RNAi results in ticks using the mosquito sequence. Interestingly, as in previous subolesin RNAi experiments with homologous and heterologous dsRNA sequences in *I. scapularis* and *A. americanum* (de la Fuente et al. 2007c) and with vacuolar adenosine triphosphatase in coleopteran insects (Baum et al. 2007), the effect of subolesin knockdown in ticks was more pronounced with homologous tick sequences than with the mosquito sequence. As noted by Gordon and Waterhouse (2007) and de la Fuente et al. (2008b), these results suggest that the RNAi process is likely to be very selective in arthropods.

The results of the sequence analysis and RNAi experiments provided evidence that the mosquito subolesin ortholog antigen may be protective against insect and tick infestations. We tested this hypothesis by conducting experiments to evaluate the protective capacity of mosquito subolesin ortholog protein antibodies on mosquitoes, sand flies, and ticks.

For mosquitoes and sand flies, the results showed evidences of the effect of antimosquito subolesin ortholog protein antibodies on insect fecundity and/or survival. Subolesin-based vaccines, as with other candidate antiarthropod vaccines, are based on the generation of antigen-specific antibodies that are deleterious to arthropod vectors during feeding (de la Fuente et al. 1998). Therefore, although the results of these studies are preliminary, they are encouraging because mosquitoes and sand flies ingest small amounts of blood as compared with ticks and are therefore exposed to less potentially detrimental host antibodies during feeding.

In ticks, the results demonstrated the protective capacity of the recombinant mosquito antigen against *I. scapularis* infestations in sheep, while the results of the vaccination trials on *A. americanum* and *R. sanguineus* infestations failed to show a protective capacity of the mosquito antigen. The variable response of individual hosts to vaccination and tick infestation is common in vaccine trials (de la Fuente et al. 1998; García-García et al. 2000; Almazán et al. 2005b) and may have affected the results with the mosquito subolesin ortholog antigen. Nonetheless, the efficacy of recombinant tick and mosquito subolesin ortholog proteins was similar for the control of *I. scapularis* adult infestations in sheep. This result was interesting because the *I. scapularis* tick subolesin is evolutionarily closer to mosquito ortholog than *A. americanum* and *R. sanguineus* sequences (de la Fuente et al. 2006b), which may have influenced vaccination efficacy with the mosqui-

to antigen against these tick species. Additionally, physiological differences not related to subolesin sequences such as the amount of blood ingested during feeding and antibody proteolysis in the gut may affect the effect of vaccination in ticks. Collectively, these results provide evidence that, while immunization of hosts with the mosquito subolesin ortholog protein may protect against tick infestations, the vaccine may be less effective against evolutionarily distant tick species. However, vaccine trials with greater number of animals per group will be needed to fully address these issues.

In this research, we choose *P. pastoris* for the expression of recombinant mosquito subolesin ortholog because this system has been shown to produce high yields of tick immunogenic protective antigens (Canales et al. 1997, 2008; García-García et al. 2000). However, it is not known if the native subolesin protein is glycosylated. Subsequently, glycosylation of recombinant proteins by *P. pastoris* may affect the immunogenicity of the antigen and the results of vaccination experiments. To address this question, experiments are in progress to evaluate the recombinant protein expressed in *Escherichia coli*.

The conservation of subolesin ortholog sequences among arthropod vectors and vertebrate hosts may raise the question of safety when using subolesin for immunization with the potential of inducing autoimmune responses damaging to the host. However, it is expected that the antibody response would be primarily directed against non-self epitopes thus reducing the possibility of detrimental effects to the host. Additionally, immunization with intracellular proteins has been effective in ticks and other invertebrate organisms and suggests a low risk to induce autoimmune responses in vertebrate hosts (Elad and Segal 1995; Almazán et al. 2005a, b; de la Fuente et al. 2006b).

Tick subolesin was shown by RNAi or immunization with the recombinant protein to reduce *A. marginale* and *A. phagocytophilum* infection levels in ticks, thus suggesting an added advantage of reducing the vector capacity of ticks for these pathogens (de la Fuente et al. 2006c). The use of subolesin ortholog proteins in vaccines may target pathogen development and transmission (not addressed here), as well as vector survival and fertility (addressed in these experiments).

The results of the experiments reported herein provide the first evidence that development of multiple arthropod vector vaccines can be possible using subolesin orthologs but multiple antigens may be required to produce an effective vaccine. Although promising, several experiments need to be done to prove the potential use of subolesin orthologs as protective antigens. Among these experiments are (1) to vaccinate experimental hosts such as mice with recombinant mosquito and tick antigens to evaluate the effect on survival and fecundity of feeding mosquitoes, (2) to evaluate the efficacy of recombinant mosquito antigen

against other tick species using a larger number of animals, (3) to compare the efficacy of recombinant mosquito and tick subolesin ortholog proteins alone and in combination for the control of mosquito, sand fly, and tick infestations and pathogen transmission, and (4) to compare the efficacy of recombinant subolesin produced in *P. pastoris* and *E. coli*. Finally, subolesin ortholog sequences and their protective efficacy should be characterized in other vector species of public and veterinary health importance such as fleas, lice, black flies, tsetse flies, and triatomine bugs.

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