Title.

Development of vaccines against *Ornithodoros* soft ticks: an update.

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Abstract.

Ticks are parasites of great medical and veterinary importance since they are vectors of numerous pathogens that affect humans, livestock and pets. Among the argasids, several species of the genus *Ornithodoros* transmit serious diseases such as tick-borne human relapsing fever (TBRF) and African Swine Fever (ASF). In particular, *Ornithodoros erraticus* is the main vector of these two diseases in the Mediterranean while *O. moubata* is the main vector in Africa. The presence of these *Ornithodoros* ticks in domestic and peridomestic environments may greatly hinder the eradication of TBRF and ASF from endemic areas. In addition, there is a constant threat of reintroduction and spreading of ASF into countries from where it has been eradicated (Spain and Portugal) or where it was never present (the Caucasus, Russia and Eastern Europe). In these countries, the presence of *Ornithodoros* vectors could have a tremendous impact on ASF transmission and long-term maintenance. Therefore, elimination of these ticks from at least synanthropic environments would contribute heavily to the prevention and control of the diseases they transmit. Tick control is a difficult task and although several methods for such control have been used, none of them has been fully effective against all ticks and the problems they cause. Nevertheless, immunological control using anti-tick vaccines offers an attractive alternative to the traditional use of acaricides. The aim of the present paper is to offer a brief overview of the current status in control measure development for *Ornithodoros* soft ticks, paying special attention to the development of vaccines against *O. erraticus* and *O. moubata*. Thus, our contribution includes an analysis of the chief attributes that the ideal antigens for an anti-tick vaccine should have, an exhaustive compilation and analysis of the scant anti-soft tick vaccine trials carried out to date using both concealed and salivary antigens and, finally, a brief description of the new reverse vaccinology approaches currently used to identify new and more effective protective tick antigens.

Keywords

Soft ticks; *Ornithodoros*; vaccines; concealed antigens; salivary antigens.

Introduction

Ticks are cosmopolitan blood-sucking ectoparasites of mammals, birds and reptiles. Ticks belong to two main families, the Argasidae (soft ticks) and the Ixodidae (hard ticks), which share common basic properties but differ in their morphology, biology and ecology. A monotypic third tick family is Nuttalliellidae, whose sole species (*Nuttalliella namaqua*) possesses features of soft and hard ticks and is considered the missing link between the main two families (Hoogstraal, 1985; Latif et al., 2012; Manzano-Román et al., 2012a).

Typically, ixodids are exophilic ticks that remain on the soil and vegetation and actively seek hosts when the season is suitable. After attachment to an adequate host they feed for several days, ingesting enormous amounts of blood and once engorged they then drop off the host returning to the soil, where they moult or, in the case of females, oviposit and die. By contrast, argasid ticks tend to be endophilic/nidicolous parasites. In the natural environment they live inside the nests and burrows of their hosts and in synanthropic environments they colonize animal facilities and human dwellings, where they remain protected from adverse climatic conditions and have regular access to host blood. Most argasids are fast feeders (they feed in less than one hour) ingesting a relatively small amount of blood per meal and adult specimens can feed and reproduce repeatedly. Argasids are very resistant to starvation and can even survive for several years without feeding (Mans and Neitz, 2004; Oleaga et al., 1990; Sonenshine, 1992; Vial, 2009).

Ticks are parasites of great medical and veterinary importance because they are the vectors of numerous pathogens including viruses, bacteria, protozoa and helminths, all of which affect humans, livestock and pets (de la Fuente et al., 2008; Jones et al., 2008). In addition, their feeding can cause direct damage to their hosts such as significant blood loss, paralysis, toxicosis, irritation, allergy and tissue destruction (Jongejan & Uilenberg, 2004).

Regarding the pathogens transmitted by argasids, these are mainly viruses and bacteria. Among the currently recognized viral diseases transmitted by soft ticks, African Swine Fever (ASF) is particularly important. In addition, most of the bacteria transmitted by argasids are *Borrelia* species that cause relapsing fever in humans (Manzano-Román et al., 2012a).

There are two types of human relapsing fever: the epidemic or louse-borne type, caused by *Borrelia recurrentis* and transmitted by the human body louse *Pediculus humanus*, and the endemic or tick-borne (TBRF) type caused by many different *Borrelia* species, which are all of them transmitted by soft ticks of the genus *Ornithodoros*. TBRF is characterized by episodes of recurring fever and non-specific symptoms such as headache, myalgia, arthralgia, chills and abdominal pains. TBRF is highly endemic in some east African countries, namely

Tanzania, where it affects the 6.4% of population and is responsible for up to 436 per 1,000 perinatal mortality rates. In Eurasia and the Americas TBRF is more sporadic affecting mainly humans who enter caves, ruins, or animal shelters infested with soft ticks (Cutler, 2006, 2010; Diatta et al., 2012; Rebaudet and Parola, 2006).

ASF is caused by the African swine fever virus (ASFV), which is the only known DNA virus with an arthropod vector (Penrith et al., 2013). ASF is a haemorrhagic fever disease of pigs with mortality rates approaching 100 %. It causes major economic losses, threatens food security and limits pork production in affected countries. The disease was originally confined to Africa until it spread to Europe in the middle of the last century and later also to South America and the Caribbean. In Europe (except Sardinia) and the Americas the infection was eradicated *via* drastic control and eradication programs. However, in 2007 the disease was introduced into Georgia, from where it rapidly spread to neighbouring Trans-Caucasian countries, the Russian Federation and Eastern Europe, and then recently into the European Union (OIE WAHID, visited December 4th 2014). There is no vaccine against ASFV and this limits the options for disease control (Costard et al., 2013; Sanchez-Vizcaíno et al., 2012, 2015).

As mentioned above, these two diseases -TBRF and ASF- are transmitted by soft tick species of the genus *Ornithodoros*. In particular, *O. moubata* is their main vector in Africa and *O. erraticus* is their main vector in the Mediterranean Basin (Costard et al., 2013; Cutler, 2010). Moreover, *O. erraticus* is the type species of the *O. erraticus* complex, which includes among others several species such as *O. alactagalis*, *O. asperus*, *O. pavlovskyi*, *O. tartakovskyi*, *O. tholozani* and *O. lahorensis*. These species are distributed throughout the Middle East, the Caucasus and the Russian Federation, where ASF is currently spreading. Although not yet experimentally proved, these species are suspected to be competent vectors for ASFV; should this be the case, they could have a tremendous impact on the transmission and long-term maintenance of ASF in this broad region (EFSA panel, 2010a, 2010b, 2010c).

The presence of *Ornithodoros* ticks in domestic and peridomestic environments is an enormous pitfall in the eradication of TBRF and ASF from endemic areas. In addition, the presence of *Ornithodoros* populations also worsens the constant threat of reintroduction, spreading and maintenance of ASF into countries from where it has been eradicated, such as Spain and Portugal, or where it was never present (Boinas et al., 2014; Costard et al., 2013; Sanchez-Vizcaíno et al., 2012, 2015; Wilson et al., 2013). Accordingly, elimination of these ticks from at least synanthropic environments would be of great help in the prevention and control of the diseases they transmit.

The aim of the present paper is to offer a brief overview of the current status in control measure development for *Ornithodoros* soft ticks, paying special attention to development of vaccines against *O. erraticus* and *O. moubata*.

Methods for tick control.

Control of tick infestations and tick-produced damages represent a multifaceted problem that remains unsolved since none of the control methods applied to date has been fully effective against all ticks (Manzano-Román et al., 2012a). The control of tick populations has been essentially based on the use of chemical acaricides (arsenicals, chlorinated hydrocarbons, organophosphates, carbamates and synthetic pyrethroids). However, these products have severe drawbacks such as environmental pollution, the contamination of animal products and the selection of resistant tick strains, which greatly reduces the usefulness and average useful life of the agent on the market (George et al., 2008; Ghosh et al., 2007; Graf et al., 2004; Guerrero et al., 2012). In addition, the use of acaricides against *Ornithodoros* ticks is inefficient because of their nidicolous/endophilic lifestyle, which means that it is not possible to ensure that these agents will reach all places where these parasites hide (Astigarraga et al., 1995).

The problems associated with the use of acaricides together with the high costs involved in the development and commercialization of new chemical agents (Guerrero et al., 2012) have encouraged the quest for alternative methods for tick control, including anti-tick vaccines and bio-control with agents such as parasitoids, predators and entomopathogenic organisms, mostly fungi, bacteria and nematodes (Samish et al., 2008).

Among these bio-control agents, the only ones tested against soft ticks to date have been entomopathogenic fungi (Samish et al., 2008). These organisms, and particularly the species *Beauveria bassiana* and *Metarhizium anisopliae*, have been shown to be effective against ticks in several laboratory and field studies (Fernandes and Bittencourt, 2008; Ment et al., 2013; Polar et al., 2008; Ren et al., 2014). However, such studies have focused almost exclusively on the control of ixodid ticks and have neglected argasid ticks. There are some exceptions to this rule, such as the works of Sewify and Habib (2001) and Habib and Sewify (2002), who studied the pathogenic effect of *M. anisopliae* and *B. bassiana* on *Argas persicus*. These authors sprayed heavily infested poultry houses with fungal spore suspensions and observed that the argasid population disappeared in 3 weeks. There is also the work of Pourseyed et al. (2010), who obtained up to 100 % mortality in larvae and females of *Argas persicus* treated with *M. anisopliae* under laboratory conditions. Finally, also interesting are the works of Zabalgogezcoa et al. (2008) and Herrero et al. (2011), who carried out laboratory

trials showing that several isolates of *B. bassiana* and *Tolypocladium cylindrosporum* caused up to 70 % mortality in *O. erraticus* and up to 40 % mortality in *O. moubata*. Therefore, further investigations for developing entomopathogenic fungal strains as anti-argasid biocontrol agents seems to be justified (Manzano-Román et al., 2012a).

Immunological control using anti-tick vaccines offers an even more attractive method than entomopathogenic fungi for replacing and/or supplementing the use of chemical acaricides. The last decade of the twentieth century saw the advent of the first two commercial anti-tick vaccines (TickGARD® in Australia and GAVAC® in Latin America). Both vaccines were directed towards the ixodid tick *Rhipicephalus microplus* and were based on the antigen Bm86, which is a glycoprotein expressed on the luminal membrane of midgut epithelial cells, hidden from the host's immune system and consequently considered a concealed antigen (Willadsen et al., 2008).

Although with variable efficacy, the application of these two vaccines during the last two decades has shown that host vaccination is a suitable and sustainable method for the control of tick populations. The implementation of anti-tick vaccines in integrated control programmes permits a reduction in the use of acaricides, which in turn decreases treatment costs. Reduced acaricide use also decreases the likelihood of acaricide resistance development, thus prolonging the average commercial life of acaricide agents (de la Fuente et al., 2007; Guerrero et al., 2012; Vargas et al., 2010; Willadsen, 2008).

Despite this success and the ensuing intensive investigation carried out in this field during the last twenty years (especially in ixodid ticks), the development of new and more effective anti-tick vaccines has been slower than expected and no new anti-tick vaccine has yet been commercialized. Several economic reasons have contributed to this delay but the main reason has been the difficulty involved in identifying new and highly protective tick antigens (Guerrero et al., 2012; Willadsen, 2008).

As a result, at present there is only a limited repertoire of vaccine candidate antigens and these come mostly from hard ticks (Guerrero et al., 2012; Merino et al., 2013; Parizi et al., 2012; Willadsen, 2008). Regarding soft ticks, the number of candidate antigens for vaccine development is even lower than for hard ticks. Thus, there is an urgent need to identify new tick protective antigens to increase the chance of developing successful anti-tick vaccines.

Desirable attributes in candidate antigens for the development of anti-tick vaccines.

There is a fair degree of consensus regarding the main attributes that an ideal antigen for an anti-tick vaccine must have (de la Fuente et al., 2011; de la Fuente, 2012; Flower et al.,

2010; Guerrero et al., 2012; Maritz-Olivier et al., 2012; Moreno-Cid et al., 2013; Parizi et al., 2012; Wikel, 2013). These attributes would be as follows.

First, the target antigen must be easily accessible to the host immune effectors (i.e., antibodies) ingested during blood feeding. Accordingly, secreted salivary proteins (exposed antigens) and membrane proteins expressed on the luminal surface of midgut cells (concealed antigens) could be suitable candidate antigens. In addition, since host immunoglobulins can pass from the gut lumen to the haemolymph (Chinzei and Minoura, 1988; Nuttall et al., 2006) other concealed antigens from internal organs bathed by haemolymph would also be suitable. Salivary exposed antigens seems quite attractive since these antigens elicit an immune response during tick infestations, and a vaccine based on such antigens would prime a vaccinated animal's immune response against tick infestation. However, some authors have expressed some reserve regarding salivary exposed antigens since the co-evolution of the tick-host interaction has likely led to the development by ticks of means to circumvent the host immune system's response to exposed antigens (Brake and Pérez de León, 2012). In this sense, "silent" salivary antigens might be more adequate because are not subjected to selective pressure by the host immune system and ticks would have not evolved mechanisms to protect them. These would include all the tick secreted salivary antigens that are not recognized by host immunity upon repeated exposure to ticks, but that can be recognized by a vaccine-induced response, as was observed for the *Ixodes scapularis* sialostatin L2 (Kotsyfakis et al., 2008). This type of antigen would allow the development of dual-action vaccines, in which tick infestations on vaccinated animals would act as boosting antigen doses (Trimnell et al., 2002).

Second, the antigen should have a critical function in the tick such that the disruption of that function would lead either to lethality or reduced fecundity at levels that impact the tick population.

Third, it would be also desirable candidate antigens encoded by unigenes or by low copy-number genes not included in any family. The aim is to prevent redundant family members from taking up the function of the targeted member and favour specific blockage of crucial functions in ticks.

Fourth, the antigen should share conserved epitopes among several tick species and, ideally, among other arthropod vectors to protect against multiple vector infestations.

Finally, it would be also very appropriate that the protective antigen could reduce the vector capacity for the transmission of vector-borne pathogens.

None of the candidates currently available fulfil all these requirements and the most effective ones meet only two or three criteria. For example, the Bm86 glycoprotein and the subolesin/akirin orthologues are both examples of concealed antigens; they are also highly

conserved in different tick species, and they also reduce vector capacity for the transmission of pathogens (de la Fuente et al., 2011).

Taking the above-mentioned concepts into consideration, a number of current research programs are prioritizing the identification of membrane-associated or membrane-bound antigens that are expressed in the midgut or ovary and that share cross-reacting epitopes (due to sequence similarity) with proteins secreted into the saliva. These antigens would be the basis for dual-action vaccines and these vaccines are considered to be the most promising ones (Guerrero et al., 2012). The primary example of this kind of cross-reacting dual action antigen is the *Rhipicephalus appendiculatus* cement antigen 64TRP (Trimnel et al., 2002, 2005). A second example of potential dual-action antigen would be the *R. microplus* antigen 2, which is a gut antigen sharing sequence similarity to a secreted homolog in salivary glands (Guerrero et al., 2012; Rachinsky et al., 2008). This antigen may be a dual action candidate since it may elicit cross-protective antibodies that might recognize the salivary homolog as well due to conserved epitopes.

Consistent with the above priority, the gut transcriptomes and proteomes from some ixodid ticks have begun to be analysed, and those from *Dermacentor variabilis* (Anderson et al., 2008) and *R. microplus* (Heekin et al. 2013; Kongsuwan et al. 2010; Popara et al., 2013; Rachinsky et al., 2008) have recently been published. Regarding argasid ticks, no gut transcriptome or proteome has been published to date, although the sialomes from four species of this family are already available (Díaz-Martín et al., 2013a; Francischetti et al. 2009), and this may serve as a reference in the search for antigens for dual-action vaccines.

Update on the development of vaccines against ornithodoros soft ticks.

To date, studies aimed at developing vaccines against soft ticks have been notably less abundant than the focused on hard ticks and have addressed just a few species belonging to the genera *Ornithodoros* and *Argas* (Table 1). Regarding *Argas* sp., the studies available include the work of Dusbábek et al. (1990) on *Argas polonicus* and the works of Sayed et al. (2001, 2004) on *Argas persicus*. Regarding *Ornithodoros* sp., the studies available are the work of Chinzei and Minoura (1988) and Salat et al. (2010) on *O. moubata*, the work of Need and Butler (1991) on *Ornithodoros turicata* and *Ornithodoros talaje*, and the studies carried out by our own team on *O. erraticus* and *O. moubata*. In these contributions, the protective efficacy of both salivary and concealed antigens has been examined.

Concealed antigens.

The first study with this type of antigen in soft ticks was performed by Chinzei and Minoura (1988), who immunized rabbits with egg vitellin and obtained a protective response that reduced female fecundity by 50 %. Most likely, the protective mechanism would have been exerted by host antibodies specific to vitellin/vitellogenin passing from the tick midgut lumen to the haemolymph, where they would have bound to haemolymph-borne vitellogenin thus blocking vitellogenin uptake by oocytes in the ovary (Ben-Yakir, 1989; Chinzei and Minoura, 1988; Gudderra et al., 2002; Horigane et al., 2010; Kopacek et al., 2000).

Soon after, Need and Butler (1991) immunized CD1 mice with protein extracts from midgut and from the remaining internal organs obtained from both *O. turicata* and *O. talaje* ticks. The larvae of these two species were allowed to feed on vaccinated mice and while no effect on the larvae of *O. turicata* was observed, the larvae of *O. talaje* had an increased mortality rate of up to 29 %. Since host antibodies (mainly IgG1) were detected in the haemolymph of the larvae of both species, the authors attributed the protective response against the long feeding *O. talaje* larvae to cellular immune effectors. Although this observation is correct, the role of antibodies in conferring protection should not be underestimated. Protection may have been associated to higher antibody quality (affinity vs. avidity) instead to higher antibody titre (Vidarsson et al., 2014) or higher antibody exposition as a consequence of prolonged feeding behaviour. Moreover, protection could have been greatly determined by the particular epitopes (protective or not) that were actually presented and recognized by the host immune system in each antigen preparation. A real example of this is commented below (Manzano-Román et al., 2015).

In 1995, our group began the studies on soft-tick concealed antigens by immunizing pigs with soluble extracts of haemolymph, synganglion, coxal glands and midgut from *O. erraticus* (Astigarraga et al., 1995). In these experiments, we did not observe any adverse effect on *O. erraticus* specimens fed on the vaccinated animals. The lack of protection was attributed to the unwanted removal of the membrane proteins from the antigenic extracts during their preparation procedure since, according to the criteria described before, membrane proteins (and particularly those expressed on the luminal surface of enterocytes) would have been the most adequate vaccine candidates.

Subsequently, Manzano-Román (2002) confirmed the above-mentioned hypothesis as he observed the following. Extracts of membrane proteins from the midgut epithelial cells of *O. erraticus* induced protective responses in pigs, rabbits and mice that reduced females feeding and fecundity by up to 50 % and, additionally, caused up to 80 % mortality in nymphs fed on vaccinated animals during the first 72 hours post-feeding. The antigen responsible for protection was a 45 kDa protein (Oe45) expressed on the luminal surface of midgut epithelial

cells between 24 and 72 hours post-feeding. The protective mechanism involved the fixation and activation of the host complement system onto the membrane of enterocytes by anti-Oe45 antibodies. This caused enterocyte lysis and midgut damage in a similar way to what was observed for the commercial anti-tick vaccines based on the Bm86 antigen (Manzano-Román et al., 2006, 2007).

The high level of protection achieved with the enterocyte membrane antigens of *O. erraticus* encouraged us to test the protective potential of a similar antigenic extract from *O. moubata* (García Varas, 2004). With this species, the protective effect was lower and essentially consisted of a 40 % decrease in feeding and reproduction without affecting tick survival. At the time, the absence of tick lethality was attributed to the fact that *O. moubata* secretes coxal fluid while it is still feeding whereas *O. erraticus* does so after detachment. Accordingly, in *O. moubata* the ingested blood could reach the midgut ceca in a sufficiently concentrated form to prevent antibodies and complement factors from diffusing to the luminal surface of the enterocytes and reaching their target antigen (García Varas, 2004).

Finally, in two recent studies our team investigated the protective value of the *O. erraticus* and *O. moubata* subolesin orthologues. Subolesin was first discovered as a protective antigen in *Ixodes scapularis* (Almazán et al., 2003). Later, it was demonstrated that subolesin is an orthologue of insect and vertebrate akirins, which are evolutionarily conserved proteins that function as transcription factors in the regulation of gene expression thus affecting numerous processes such as feeding, fertility and tissue development (Galindo et al., 2009; Goto et al., 2008). Moreover, subolesin was found to be functionally involved in innate tick immunity to pathogens and in the infection and multiplication of some pathogens such as *Anaplasma phagocytophilum*, *A. marginale*, *Babesia bigemina* and *Borrelia burgdorferi* (de la Fuente et al., 2013). Subolesin gene knockdown by RNAi and vaccination with recombinant subolesin/akirin has a profound effect on tick infestations reducing tick numbers, weight and oviposition, as well as on tick infection with different tick-borne pathogens. Additionally, subolesin/akirin orthologues share common structural and protective epitopes in different arthropod vector species including hard ticks, mosquitoes, sand flies, poultry red mites and sea lice. This highlights the potential of subolesin/akirin-based vaccines for the control of vectors and vector-borne pathogens (de la Fuente et al., 2011, 2013; Moreno-Cid et al., 2013).

Regarding the protective value of subolesin against soft ticks, we observed that knocking down the subolesin gene orthologues in *O. erraticus* and *O. moubata* by RNAi inhibited tick oviposition by more than 90 % without affecting tick feeding and survival. However, vaccination with the corresponding recombinant proteins induced robust but low

protective humoral responses (5 % - 24.5 % reduction in oviposition) (Manzano-Román et al., 2012b).

Trying to explain the reason for this low degree of protection, it was later observed that vaccination with whole subolesin recombinants induced non-protective antibodies mainly directed to immunodominant linear B-cell epitopes located on highly structured regions of the subolesin protein. Structured regions are likely unrelated to the subolesin biological activity, while the unstructured/disordered regions of the protein, which are assumed to contain the functional site, were not recognized by the vaccine-induced antibodies. Subsequently, four synthetic peptides from the unrecognized/disordered regions of the *Ornithodoros* subolesin sequences were designed, synthesized and coupled to keyhole limpet haemocyanin (KLH). Vaccination with KLH-peptide conjugates induced the synthesis of specific antibodies that recognized linear B-cell epitopes located on the unstructured loops of the subolesin proteins and provided up to 70.1 % and 83.1 % vaccine efficacies against *O. erraticus* and *O. moubata*, respectively (Manzano-Román et al., 2015). These results demonstrated that the protective effect of subolesin-based vaccines was highly dependent on the particular epitope being recognized by antibodies and strongly suggest that the biological activity of subolesin is exerted through its unstructured regions, in accordance with recent findings by de la Fuente et al. (2013), Merino et al. (2013) and Moreno-Cid et al. (2013).

Overall, these results contribute to our understanding of the mechanism of protection of subolesin-based vaccines. They point to additional protective peptides for inclusion in the array of candidate antigens and expand the range of target organisms for subolesin/akirin-based vaccines against soft ticks.

Salivary antigens.

In general, the few vaccine trials performed to date with salivary antigens have reported highly variable protection efficacies. Need and Butler (1991) reported 16 % mortality in *O. talaje* larvae fed on CD1 mice vaccinated with a salivary gland extract (SGE) from that species, similar to the mortality rate observed in mice immunized by natural contact. More recently, Salat et al. (2010) evaluated the protective potential of recombinant OmC2 cystatin from *O. moubata* in C3H/HeN mice and found 15 % increase in mortality and 39 % reduction in the feeding of *O. moubata* nymphs-1 fed on vaccinated mice.

Regarding the studies on soft tick salivary antigens carried out by our team, these were begun by vaccinating pigs with SGE obtained from *O. erraticus* and *O. moubata* (Astigarraga et al., 1995).

Vaccination with *O. erraticus* SGE induced highly variable protective responses, the best of them attaining a 50 % reduction in the feeding and fecundity of females. These results clearly showed that at least in *O. erraticus* it was possible to induce protective responses using salivary antigens. The protection was associated to several silent antigens of the SGE (namely, three proteins of 70, 50 and 20 kDa) that were specifically recognized by the humoral immune response of the SGE-vaccinated pigs but not recognized by the humoral response of unprotected pigs sensitised by natural parasite contact (Astigarraga et al., 1995). Subsequently, to identify antigens able to induce uniform humoral protective responses, Manzano-Román (2002) purified these three proteins from the SGE and tested them individually in new vaccine trials. All pigs vaccinated with either the 70 kDa or 50 kDa antigens developed antibody responses that specifically recognized the inducing antigen. However, these responses did not protect precluding the usefulness of these antigens as vaccine candidates. By contrast, only a part of all the pigs vaccinated with the 20 kDa antigen were able to develop a specific antibody response to it. This response was protective and reduced female feeding and subsequently female fertility by up to 50 %. However, most pigs vaccinated with the 20 kDa antigen were unable to develop a detectable antibody response to this antigen (regardless of the dose, the adjuvant used or the mode of administration) and none of these pigs were protected. From these observations it was concluded that protection can be achieved after the antibody-mediated neutralization of a particular silent salivary antigen (such that of 20 kDa). This antigen seems to be strongly protected from being recognized by the host immune system, as suggested by the difficulties encountered in inducing humoral responses to it in all the vaccinated animals thus limiting its efficacy as vaccine candidate (Manzano-Román, 2002). The disadvantages and limitations of these three salivary antigens avoided further sequence and functional characterization. Thus, their sequences and functions as well as how the 20 kDa antigen evades its recognition by the immune system remain unknown.

Regarding *O. moubata*, pig vaccination with SGE induced very homogeneous protective responses causing 60 % to 70 % reductions in adult tick feeding and female oviposition. All vaccinated animals recognized a protein of 44 kDa (hence the name Om44), which was never recognized by the host immune system during natural parasite contact (Astigarraga et al., 1995).

Subsequent studies revealed that purified Om44 induced in pigs and rabbits a protective response that inhibited tick feeding by up to 54 % and the protective effect increased with successive *O. moubata* infestations (García-Varas et al., 2010). Thus, Om44 must be a silent salivary antigen according to the concept introduced by Kotsyfakis et al. (2008). Functional characterization of Om44 showed that it is an antagonist ligand of host P-

selectin. Accordingly, Om44 probably inhibits the host haemostatic and inflammatory response triggered at the tick bite lesion by the interaction between P-selectin on activated endothelial cells and platelets and its receptor on leucocytes, the PSGL-1 molecule (Cleator et al., 2006; García-Varas et al., 2010). Since this response could prevent tick feeding, blocking the P-selectin/PSGL-1 interaction at the tick bite site seems to be critical for *O. moubata* to complete its feeding. These results represented the first evidence of a new anti-haemostatic mechanism of hematophagous vectors and led Om44 to be assigned the place of an important candidate for vaccine development.

The identification of Om44 was repeatedly attempted by mass spectrometry without success. Mass spectrometric data clearly showed that Om44 was not an orthologue of already established P-selectin ligands including PSGL-1 and pentraxin 3. However, the results of some of the analyses performed suggested that Om44 could be an isoform of enolase (García-Varas et al., 2010; Perez-Sánchez et al., 2010). Since significant amounts of secreted enolase had been found in the saliva of *O. moubata* ticks (Díaz-Martín et al. 2013a), this protein was cloned and functionally characterized. The results of this study showed that secreted salivary enolase acts as a profibrinolytic receptor of host plasminogen confirming that it was not the identity of Om44. Additionally, vaccination of rabbits with recombinant enolase induced partial protective responses (18 % reduction in oviposition and up to 20 % nymphal mortality) differed from those induced by Om44 (Díaz-Martín et al. 2013b).

In order to identify Om44, more recently self-assembled protein microarrays from an *O. moubata* salivary gland expression library were screened using P-selectin and anti-Om44 polyclonal serum as probes. The results of these experiments indicated that Om44 was a salivary secreted phospholipase A₂ (GenBank KC908103.1) that binds to P-selectin through protein-protein interactions without the involvement of glycan moieties (Díaz-Martín, 2014; Manzano-Román et al., 2012c).

Moreover, random sequencing of the above expression library allowed us to identify two additional anti-haemostatic salivary proteins of *O. moubata*, in particular an apyrase (GenBank KC908110.1) and a disaggregin (named as mougrin) that is orthologous to the savignygrin of *O. savignyi* (GenBank KC908105.1). Vaccination of rabbits with the recombinant forms of these three salivary proteins (elicited individual protective efficacies between 27 % and 44 % (table 1). These results indicate that *O. moubata* salivary anti-haemostatics are suitable antigen candidates for the development of anti-tick vaccines that deserve further studies (Díaz-Martín, 2014).

Overall, these results confirm that soft tick salivary antigens are able to induce protective immune responses when administered individually. Such protection essentially

consists of the partial inhibition of blood feeding and subsequent reduction in female fertility, most likely as a consequence of the antibody-mediated loss of function of the target antigen at the tick-host interface.

Although exploring additional adjuvants and immunization protocols would probably increase the protective efficacies of these antigens, our opinion is that a main factor limiting the protection achieved with these vaccines (based on recombinant salivary antigens administered individually) is the complexity, multiplicity and functional redundancy of tick saliva composition, which would compensate the vaccine-mediated loss of function of the target antigen with non-targeted salivary proteins exerting redundant functions (Chmelar et al., 2012; Kazimírová and Štibrániová, 2013; Wikel, 2013; Willadsen, 2008). Hence, the use of vaccines formulated with several target antigens would likely increase the protective efficacy of such vaccines.

In addition, the identification of the protective epitopes on those antigens would allow the construction of chimeric recombinant antigens in order to simultaneously target redundant anti-defensive mechanisms in ticks. Accordingly, the results described here could serve as a starting point to search for protective epitopes in immunogenic proteins from argasids (such as phospholipase A₂, apyrase and mougryn) that have functional orthologues in ixodids to identify similar or conserved protective epitopes. The identification and inclusion in a chimeric recombinant antigen of such epitopes would offer the possibility of obtaining broader-spectrum vaccines that could be useful for the control of a greater number of vectors and by extension the pathogens they transmit (de la Fuente, 2012; Merino et al., 2013; Parizi et al., 2012; Prudencio et al., 2010).

According to Sikic and Carugo (2010), the increasing potential of new computational tools for selecting proteins with specific functions can aid in large-scale analyses for the identification of potential new antigen targets for vaccines. This could also be applied to the identification and designing of synthetic epitopes conserved in redundant protein families.

Future prospects and new strategies for the identification of novel protective antigens.

In his review on anti-tick vaccines, Willadsen (2008) analysed the three main types of experimental approaches traditionally used for identifying useful vaccine antigens in ticks: i.e., the immunological one, based on studying antigens that elicit an immune response; the functional one, based on identifying tick factors important for the parasite's function and survival and then evaluating them as potential vaccine antigens; and biochemical fractionation,

based on evaluating progressively simpler protein mixtures by host vaccination and parasite challenge trials.

Currently, the increasing availability of tick genomic, transcriptomic and proteomic sequences in public data bases (namely: VectorBase, The Gene Index Project, CattleTickBase) together with the development and application of new technologies of large-scale analysis (NGS, RNAseq, microarrays, etc.) is driving the rapid transition from the above-mentioned traditional approaches for protective antigen identification to new strategies based on reverse vaccinology, which constitute a very important part of the emerging field of vaccinomics (Aljamali et al., 2009a, 2009b; de la Fuente and Merino, 2013; Flower et al., 2010; Guerrero et al., 2012; Manzano-Román et al., 2013; Maritz-Olivier et al., 2012; Oliveira et al., 2013; Ribeiro and Arcá, 2009; Ribeiro et al., 2011, 2012).

These holistic approaches analyse the interactions between hosts, ticks and pathogens, obtaining genomic, transcriptomic, proteomic, immunological and metabolic information (systems biology) that is later integrated and analysed *in silico* to perform a theoretical, hypothesis-driven selection of candidate vaccine antigens (Nakaya et al., 2012; Six et al., 2012). The resulting candidate antigen list can then be progressively reduced by carrying out relatively simple and cost-effective experimental assays in order to select those with the highest protective potential. One of these assays is the RNAi-mediated gene knockdown (de la Fuente et al., 2010; Manzano-Román et al. 2012b, 2012c). In spite of its limitations, which include the potential incongruity between RNAi loss of function phenotypes and vaccine efficacy of the involved proteins *in vivo* (Nijhof et al., 2007), RNAi has demonstrated a great potential in the context of screening genomic and transcriptomic data from arthropod ectoparasites becoming an attractive post-genomic tool to inform the selection of vaccine candidates (Marr et al., 2014). Selected candidates could then be obtained as recombinant proteins and validated as protective antigens in animal vaccination trials, and their function and protective mechanism could be characterized (de la Fuente and Merino, 2013) (Figure 1).

The application of these new strategies to soft ticks has just begun. The scant transcriptomic and proteomic data currently available are limited to the salivary glands and/or saliva of half a dozen species (Díaz-Martín et al., 2013a; Francischetti et al., 2009; Ribeiro et al., 2012) while no “omic” data from any other tissue or organ from soft ticks have been obtained.

Some available comparative analyses of the soft and hard tick sialomes indicate that the major families of salivary proteins are conserved between tick families encouraging the search for conserved protective antigens (Mans et al., 2008). Obtaining and analysing sialomes from more soft tick vectors will provide valuable data to identify new vaccine candidates including potential dual-action antigens if proved that they are secreted into saliva and so that

exposed to host in natural contact. Hence our group applied proteomics to study the saliva of *O. moubata* unveiling notable differences in the saliva composition between females and males as well as a high ratio of intracellular housekeeping proteins that were secreted to saliva by non-classical ways, which significantly increased the expected range of salivary proteins exposed to the host immune system during natural infestations (Diaz-Martín et al., 2013a). Moreover, we developed Nucleic Acid Programmable Protein Arrays (NAPPA) from an *O. moubata* salivary gland cDNA expression library to study protein interactions and functions in a high-throughput format and cell-free system. As mentioned previously, the screening of these microarrays allowed identifying some new salivary protective antigens (Manzano-Román et al., 2012b).

As highlighted in section 3, a number of current research programs are prioritizing the identification of protective antigens that are expressed in the midgut membranes of hard ticks. This should be also a priority for soft ticks but the absence of argasid genomes and argasid midgut transcriptomes and proteomes is precluding the application of reverse vaccinology strategies to the identification of intestinal protective antigens. Therefore, obtaining and analysing midgut transcriptomes and proteomes from soft tick vectors is urgently needed. This information will facilitate basic knowledge about the biology of these parasites and their relationship with hosts and tick-borne pathogens. This information will also permit comparisons to ixodid midgut transcriptomes and proteomes and probably the identification of conserved antigens in both tick families allowing the selection of target antigens. The combination of key midgut and salivary antigens will facilitate development of wide-range highly effective vaccines for the control of ticks and the diseases they transmit.

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Figure Caption.

Figure 1. Reverse vaccinology approaches for the identification and validation of soft tick protective antigens. When searching for suitable vaccine candidates a number of desirable attributes might be taken into consideration. Currently, tick midgut and salivary glands are being prioritized as the most appropriate sources of protective antigens. The transcriptomic, proteomic and immunological data from the targeted organ or tissue are integrated and analysed *in silico* performing a theoretical selection of vaccine candidates. The resulting candidate antigen list can be progressively reduced by applying experimental tools (i.e., RNAi) that may inform on antigens with high protective potential. Selected candidates are obtained as recombinant proteins and validated as protective antigens in animal vaccination trials.

^a, Salivary antigens from females are prioritized in order to target feeding (and fecundity) in this developmental stage.

^b, for midgut antigens, vaccine-derived deleterious effects can be independent of the antigen function if the antigen acts as an anchor for host immune effectors such as antibodies and complement (i.e., Bm86).

Figure 1
Figure 1

Table 1

Table 1. Vaccine trials using concealed and salivary antigens from soft ticks.

Species	Antigen name	Protein identity	Protein type	Protein function	Host	PROTECTION	REFERENCE
Concealed antigens							
<i>Argas polonicus</i>	LH	-	Larval homogenate	-	Pigeon	60% reduction in engorged larvae	Dusbábek et al., 1990
<i>Argas persicus</i>	DNA from eggs	-	-	-	Chicken	75% - 89% rejection of feeding ticks	Sayed et al., 2004
<i>O. erraticus</i>	Haemolymph	-	Soluble fraction of tissue extracts	-	Pig	No protection	Astigarraga et al., 1995
	Synganglion	-		-			
	Coxal glands	-		-			
	Midgut	-		-			
	GME	-	Membrane proteins (insoluble fraction of midgut extract)	-	Pig	80% reduction in nymphs survival, 50% reduction in female feeding and fecundity	Manzano-Román et al., 2006
	GME	-	Membrane proteins (insoluble fraction of midgut extract)	-	Mouse	67% and 29% reduction in nymphs and larvae survival, respectively	Manzano-Román et al., 2006
	Oe45	Not determined	Native protein, purified from midgut membranes	-	Pig	21% - 27% reduction in nymphs survival, 35% - 42% reduction in female feeding and fecundity	Manzano-Román et al., 2007

	rOeSub	<i>O. erraticus</i> subolesin	Recombinant	Transcription factor	Rabbit	22% reduction in oviposition	Manzano-Román et al., 2012b
	rOmSub	<i>O. moubata</i> subolesin	Recombinant	Transcription factor	Rabbit	24.3 % reduction in oviposition	
	OE1	<i>O. erraticus</i> subolesin	Synthetic peptides	Transcription factor	Rabbit	49% reduction in fecundity	Manzano-Román et al., 2015
	OE2	<i>O. erraticus</i> subolesin				82% reduction in fecundity	
	OM1	<i>O. moubata</i> subolesin				50% reduction in fecundity	
	OM2	<i>O. moubata</i> subolesin				17% reduction in fecundity	
<i>O. moubata</i>	Egg yolk protein	Vitellin	Native	Embryonic development	Rabbit	50% reduction in oviposition	Chinzei and Minoura, 1988
	GME	-	Membrane proteins (insoluble fraction of midgut extract)	-	Mouse, rabbit, pig	40% reduction in female feeding and reproduction	García-Varas, 2004
	rOeSub	<i>O. erraticus</i> subolesin	Recombinant	Transcription factor	Rabbit	8.5 % reduction in oviposition	Manzano-Román et al., 2012b
	rOmSub	<i>O. moubata</i> subolesin	Recombinant	Transcription factor	Rabbit	5.2 % reduction in oviposition	
	OE1	<i>O. erraticus</i> subolesin	Synthetic peptides	Transcription factor	Rabbit	35% reduction in fecundity	Manzano-Román et al., 2015
	OE2	<i>O. erraticus</i> subolesin				40% reduction in fecundity	
	OM1	<i>O. moubata</i> subolesin				45% reduction in fecundity	
	OM2	<i>O. moubata</i> subolesin				60% reduction in fecundity	

<i>O. talaje</i>	Gut	-	Tissue extract	-	CD1 mouse	32% reduction in tick survival	Need and Butler, 1991
	Internal organs, not gut	-	Tissue extract	-	CD1 mouse	27% reduction in tick survival	
<i>O. turicata</i>	Gut	-	Tissue extract	-	CD1 mouse	No protection	Need and Butler, 1991
	Internal organs, not gut	-	Tissue extract	-	CD1 mouse	No protection	
Salivary antigens							
<i>Argas polonicus</i>	SGE	-	Salivary gland extract	-	Pigeon	No protection	Dusbábek et al., 1990
<i>Argas persicus</i>	SGE	-	Salivary gland extract	-	Chicken	58% - 62% rejection of feeding ticks	Sayed et al., 2001
<i>O. erraticus</i>	SGE	-	Salivary gland extract	-	Pig	Variable, < 50% reduction in female feeding and fecundity	Astigarraga et al., 1995
	70 kDa antigen	Not determined	Native, purified from SGE	Not determined	Pig	No protection	Manzano-Román, 2002
	50 kDa antigen	Not determined	Native, purified from SGE	Not determined	Pig	No protection	
	20 kDa antigen	Not determined	Native, purified from SGE	Not determined	Pig	Up to 50% reduction in female feeding and fecundity	
<i>O. moubata</i>	SGE	-	Salivary gland extract	-	Pig	60% - 70 % reduction in female feeding and fecundity	Astigarraga et al., 1995
	Om44	-	Native	P-selectin antagonist	Pig	54% reduction in tick feeding, 50% reduction in	García-Varas et al., 2010

	OmC2	Cystatin	Recombinant	Peptidase inhibitor	C3H/HeN mouse	female fecundity 39% reduction in nymph-1 feeding, 15% increase in nymph-1 mortality	Salat et al., 2010
	rOmENO	Enolase	Recombinant	Glycolytic enzyme	Rabbit	18% reduction in female fecundity, 20% increase in nymphal mortality	Díaz-Martín et al., 2013
	rOmPLA2	Phospholipase A ₂	Recombinant	Anti-haemostatic	Rabbit	23% reduction in female fertility, 11% - 32% increase in tick mortality (E = 44.2%) ^a	Díaz-Martín, 2014
	rOmAPY	Apyrase	Recombinant	Anti-platelet aggregation	Rabbit	26% reduction in female fertility, 16% increase in adult mortality (E = 27.2%) ^a	
	rOmMOU	Mougrin	Recombinant	disaggregin	Rabbit	32% reduction in female fecundity (E = 43.2%) ^a	
	SGE	-	Salivary gland extract	-	CD1 mouse	19% reduction in larvae survival	Need and Butler, 1991

^aVaccine efficacy (E) was calculated as $E = 100 (1 - S \times F)$, 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 the adjuvant only).