Targeting the tick-pathogen interface for novel control strategies

Jose de la Fuente¹,², Katherine M. Kocan¹, Consuelo Almazan³, Edmour F. Blouin¹

¹Department of Veterinary Pathobiology, Center for Veterinary Health Sciences, Oklahoma State University, Stillwater, OK 74078, USA, ²Instituto de Investigacion en Recursos Cinegeticos IREC (CSIC-UCLM-JCCM), Ronda de Toledo s/n, 13005 Ciudad Real, Spain, ³Facultad de Medicina Veterinaria y Zootecnia, Universidad Autonoma de Tamaulipas, Km. 5 carretera Victoria-Mante, CP 87000 Victoria, Tamaulipas, Mexico

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1. ABSTRACT

Ticks are ectoparasites of wild and domestic animals and humans that most notably impact global health by transmitting disease-causing pathogens. While information on the molecular interactions between ticks and pathogens that facilitate pathogen infection, development and transmission is limited, a comprehensive understanding of the tick-pathogen interface would be fundamental toward development of new and novel measures for control of both tick infestations and tick-borne pathogens. Recently, vaccine studies using key tick antigens and characterization of tick gene function by RNA interference (RNAi) have provided new information on genes that impact the tick-pathogen interface. In this review we summarize current research and prospects of tick vaccines and genetic manipulation of ticks targeted to the tick-pathogen interface. The knowledge gained from these collective studies will be fundamental toward understanding of tick-pathogen interactions and for formulation of control methods targeted at both ticks and tick-borne pathogens. Use of these molecular approaches will likely contribute to control measures that will notably reduce tick populations and tick-borne diseases in the future.

2. INTRODUCTION

Ticks are obligate hematophagous ectoparasites of wild and domestic animals and humans that are classified in the subclass Acari, order Parasitiformes, suborder Ixodida and distributed from Arctic to tropical regions of the world (1, 2). Despite efforts to implement measures to control tick infestations, ticks and the pathogens they transmit continue to be a serious constraint to human and animal health (3, 4).

Ticks are considered to be second worldwide to mosquitoes as vectors of human diseases (5) and the most important vector of diseases that affect the cattle industry worldwide (6). For example, *Ixodes* spp. vector several human pathogens, including *Borrelia burgdorferi* (Lyme disease), the closely related *B. afzelii, B. lusitaniae, B. valaisiana* and *B. garinii,* *Anaplasma phagocytophilum* (human granulocytic anaplasmosis), *Coxiella burnetii* (Q fever), *Francisella tularensis* (tularemia), *Rickettsia helvetica, R. japonica* and *R. australis,* *Babesia divergens,* as well as tick-borne encephalitis virus (TBEV), a causative agent of tick-borne encephalitis. *Boophilus* spp. are major tick pests of veterinary importance in tropical regions. Infestations with the cattle tick, *B. microplus,* economically
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Impact cattle production by causing reductions in weight gains and milk production, and by transmitting pathogens that cause babesiosis (Babesia bovis and B. bigemina) and anaplasmosis (A. marginale).

New strategies are needed for the control of ticks and tick-borne pathogens. Characterization of the molecular mechanisms that mediate tick-pathogen interactions will likely provide new targets for vaccines and genetic manipulation of ticks. Evidence that co-evolution and long-term survival of ticks and tick-borne pathogens undoubtedly involves genetic traits from both organisms including reports of pathogen ligands and tick receptors that enable pathogen infection of ticks (7, 8).

Recent advancements in tick vaccine development and RNA interference (RNAi) have been reviewed in several publications (9-15). Herein, we will extend these reviews by focusing on the application of molecular technologies targeted to the tick-pathogen interface. These technologies, in concert with other emerging ones, are needed to advance our understanding of tick-pathogen interactions and will likely provide new and novel strategies for development of improved vaccines for the simultaneous control of ticks and tick-borne pathogens.

3. TICK VACCINES AND THE TICK-PATHOGEN INTERFACE

3.1. Tick vaccines for the control of tick infestations

The feasibility of controlling tick infestations through immunization of hosts with selected tick antigens was demonstrated following the pioneering work of Allen and Humphreys (16) with the development of vaccines that reduced Boophilus spp. infestations on cattle (recently reviewed by 3, 4, 13, 14). This vaccine, based on the recombinant B. microplus Bm86 gut antigen, caused reductions in the number, weight and reproductive capacity of female ticks that fed on immunized cattle. Overall, the most notable vaccine effect was the decline of larval infestations in subsequent tick generations. Controlled field trials using Bm86-based vaccines in cattle resulted in the control of B. microplus and B. annulatus infestations and a correlation was found between the antibody titers of cattle immunized with Bm86 and tick fecundity (17). Use of the integrated approach of acaricide application and vaccination in relation to seasonal tick populations resulted in a more cost-effective and environmentally sound mean of controlling tick infestations by reducing acaricides applications (13, 14, 17, 18).

3.2. Discovery of tick vaccine antigens

The Bm86 tick vaccine antigen that was first used in commercial cattle tick vaccines was discovered from proteins in crude tick extracts after fractionation, immunization and tick challenge in cattle (19). Subsequent approaches for antigen identification included immunomapping tick antigens that elicited an antibody response in the infested hosts and the testing in vaccination experiments of tick proteins that were considered to be important for the parasite function and/or survival (3, 13).

Recently, advances in molecular biology and systems biology have provided new avenues for efficient discovery of tick vaccine antigens. The initial tick antigens selected for study were based on the assumption that cell membrane-exposed or secreted proteins would be required for antibody recognition in ticks fed on vaccinated cattle. However, tick protective antigens identified by expression library immunization and RNAi included those intracellular proteins that play important roles in tick physiology and reproduction and may therefore also be candidate vaccine antigens (20, 21). Although the mechanism by which antibodies are transported across tick cell membranes and complex with antigens is not completely understood, antibodies are specifically transported inside tick cells to interact and neutralize intracellular proteins (22, 23). Therefore, these results challenge the paradigm that vaccine antigens must be extracellular proteins and thus expand the repertoire of candidate tick vaccine antigens.

In the near future, the application of high throughput functional genomics screening will likely result in the discovery of tick vaccine antigens that include both extracellular and intracellular proteins.

3.3. Tick vaccines and the control of tick-borne pathogens

While reduction of tick infestations has been a foremost goal of tick vaccine development, an equally important impact is reduction of the transmission of medically and economically important pathogens by ticks. Immunization of cattle with Bm86 vaccines resulted in lower infestations as well as a reduction in the incidence of babesiosis and anaplasmosis in some regions (13, 14, 17, 18). Tick vaccines may therefore have a dual impact of targeting tick infestations and the incidence of tick-borne diseases by decreasing the exposure of susceptible hosts to ticks (Figure 1).

3.4. Feasibility of targeting tick proteins to reduce tick vector capacity

Recent research results demonstrated that tick vaccines also reduce tick vector capacity. The candidate antigens involved in vector capacity were those at the tick-pathogen interface found to be necessary for the infection, development and transmission of pathogens. Three proteins have been shown thus far to be involved in tick vector capacity: the tick receptor for OspA (TROSPA) that is required for colonization of I. scapularis with B. burgdorferi (8); the R. sanguineus P64 putative cement protein (24); and the tick protective antigen subolesin (25). Use of these antigens in vaccine formulations has resulted in reduced vector capacity of ticks for infection with B. burgdorferi, TBEV and A. phagocytophilum/A. marginale, respectively. Recently, Narasimhan et al. (26) demonstrated that immunity against I. scapularis salivary proteins that are expressed within 24 hours of tick attachment impaired transmission of B. burgdorferi by ticks to guinea pigs.

The feasibility of vaccinating animal host populations to decrease the prevalence of tick-borne pathogens in ticks, thus reducing the risk for pathogen
transmission to humans and animals, was recently demonstrated by Gomes-Solecki et al. (27) and Tsao et al. (28). In this research, B. burgdorferi infections in I. scapularis were reduced when ticks fed on mice immunized with the bacterial protein OspA, possibly by blocking bacterial adhesion to the tick receptor.

Collectively, these results demonstrated the feasibility of developing vaccines by targeting pathogen and tick-derived key proteins at the tick-pathogen interface for the control of tick infestations and the transmission of tick-borne pathogens (29-31) (Figure 1).

4. GENETIC MANIPULATION OF TICKS TO TARGET THE TICK-PATHOGEN INTERFACE

4.1. Methods and mechanism of RNAi in ticks

RNAi is a nucleic acid-based reverse genetic approach that results in the silencing of gene expression and allows for study of the impact of gene function on metabolic pathways (32). Small interfering RNAs (siRNAs) are the effector molecules of the RNAi pathway that is initiated by double-stranded RNA (dsRNA) and results in a potent sequence-specific degradation of cytoplasmic mRNAs containing the same sequence as the dsRNA trigger (33). Post-transcriptional gene silencing mechanisms initiated by dsRNA have been reported in all eukaryotes studied thus far, and RNAi has rapidly become an important molecular tool for functional genomics studies on a variety of organisms, as well as other applications (34).

RNAi is currently the most widely used gene-silencing technique in ticks because use of other methods of genetic manipulations has been limited. As recently reviewed by de la Fuente et al. (15), RNAi has rapidly proved to be a valuable tool for studying tick gene function, for characterization of the tick-pathogen interface and for the screening and characterization of tick protective antigens.

Four methods have been used for RNAi in ticks: (i) injection or microinjection of dsRNA into unfed or fed ticks, (ii) soaking or incubation of dsRNA with ticks, tick tissues or cells, (iii) feeding of dsRNA to ticks and (iv) virus production of dsRNA (15). The application of these methods for RNAi in ticks depends on the experimental design and objectives. Injection of dsRNA into adult ticks is the most universal method for in vivo RNAi in ticks, particularly with the possibility of generating a high number of treated individuals through inherited RNAi (35, 36). Inherited RNAi and feeding of dsRNA solutions to ticks are relatively easy ways to deliver dsRNA into immature tick stages for the study of tick-pathogen interactions (35-37). However, incubation with dsRNA solutions is probably the best approach for ex vivo and in vitro studies of gene expression and pathogen infection and multiplication in isolated tick organs and cell lines (38, 39). Virus production of dsRNA may impact the generation of transgenic ticks resistant to tick-borne pathogens (see section 4.3).

The mechanism of RNAi in ticks has not been characterized and the proteins involved in the process of RNAi have not been identified, with the exception of a B. microplus sequence that was shown to be similar to Argonaute (Ago)-2 (15). We recently proposed a model for RNAi in ticks based on current information on RNAi in Drosophila melanogaster and mosquitoes (15). Exogenous or viral dsRNA enter the cytoplasm, where it is first
processed into double stranded siRNAs about 21–23 nucleotides in length. The key protein for this specific degradation in *D. melanogaster* is Dicer-1. This RNase III-like dsRNA-specific ribonuclease contains RNase III, helicase, and PAZ (Piwi/Argonaute/Zwille) domains that are involved in protein–protein interactions, a dsRNA binding domain and a DEAD-box helicase domain. Dicer-1 then presents the siRNAs to the RNA-induced silencing complex (RISC), which incorporates the siRNAs and targets and degrades any mRNA with cognate sequences. Other protein components of RISC, such as the Ago protein family, may be required for RNAi. However, a more complete understanding of the mechanism of dsRNA-induced RNai in ticks would facilitate a better use of this genetic approach.

4.2. RNAi for the characterization of the tick-pathogen interface

Molecular characterization of the tick-pathogen interface is an emerging research area. Differential gene expression has been characterized in *Dermacentor variabilis* ovaries in response to rickettsial infection (40), in salivary glands of female *R. appendiculatus* infected with *Theileria parva* (41) and in *I. scapularis* IDE8 cells, *D. variabilis* and *B. microplus* ticks in response to infection with *A. marginale* (42). While the function of many tick genes at the tick-pathogen interface is unknown, results thus far have provided evidence that pathogens modify expression of tick genes involved in defense mechanisms, thus facilitating tick vector capacity.

While RNAi has been used primarily for study of tick gene function (15), it was also used recently to study the role of tick gene expression in infection and transmission of *B. burgdorferi* (8, 37, 43), *A. phagocytophilum* (44), and *A. marginale* (25). Silencing of key genes in the respective tick vectors resulted in reduction of the tick vector capacity for these pathogens (Table 1). However, in other RNAi experiments, gene knockdown did not affect pathogen acquisition by ticks (45) or facilitated *B. gibsoni* transmission by targeting the *Haemaphysalis longicornis* antimicrobial peptide, longicin (46) (Table 1).

Studies of the tick protective antigen, subolesin, provided evidence that tick gene expression may be targeted by RNAi to test the role of encoded proteins in tick biology and the vector capacity of ticks (Figure 2). Subolesin was recently shown by both RNAi gene silencing and immunization trials using the recombinant protein to protect hosts against tick infestations, reduce tick survival and reproduction, and cause degeneration of gut, salivary gland, reproductive tissues and embryos (35, 36, 47–50). The silencing of subolesin expression by RNAi also decreased vector capacity of ticks for *A. marginale* (25). In addition, subolesin mRNA levels were determined by real-time RT-PCR in uninfected and *A. marginale*-infected *D. variabilis* guts and salivary glands and IDE8 cultured tick cells, as well as in uninfected and *A. phagocytophilum*-infected *I. scapularis* nymphs and ISE6 cultured tick cells (51). In these experiments, subolesin was differentially expressed in *A. marginale*-infected ticks in a tissue-specific manner in which mRNA levels increased in response to *A. marginale* infection in tick salivary glands but not in the guts. Subolesin knockdown by RNAi reduced *Anaplasma* infection/multiplication only in cells in which infection increased subolesin expression, i.e. in *A. marginale*-infected *D. variabilis* salivary glands and IDE8 cells (51).

### Table 1. Summary of RNA interference experiments targeting the tick-pathogen interface

<table>
<thead>
<tr>
<th>Tick species</th>
<th>Target gene</th>
<th>Delivery of dsRNA</th>
<th>Phenotype at the tick-pathogen interface</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ixodes scapularis</em></td>
<td>TROSPA</td>
<td>Microinjection into nymphal ticks</td>
<td>Reduction in infection and transmission of <em>B. burgdorferi</em></td>
<td>8</td>
</tr>
<tr>
<td><em>Ixodes scapularis</em></td>
<td>Salp15</td>
<td>Microinjection into nymphal ticks</td>
<td>Reduction in the capacity of tick-borne <em>B. burgdorferi</em> to infect mice</td>
<td>43</td>
</tr>
<tr>
<td><em>Ixodes scapularis</em></td>
<td>Salp16</td>
<td>Microinjection into nymphal ticks</td>
<td>Reduction of <em>A. phagocytophilum</em> survival in ticks</td>
<td>44</td>
</tr>
<tr>
<td><em>Ixodes scapularis</em></td>
<td>Salp14</td>
<td>Microinjection into nymphal ticks</td>
<td>Reduction in mRNA levels but no effect on acquisition of <em>A. phagocytophilum</em> and <em>B. burgdorferi</em></td>
<td>45</td>
</tr>
<tr>
<td><em>Ixodes scapularis</em></td>
<td>Isac</td>
<td>Nymph capillary feeding</td>
<td>Reduction in tick infection by <em>B. burgdorferi</em></td>
<td>37</td>
</tr>
<tr>
<td><em>Dermacentor variabilis</em></td>
<td>Subolesin (4D8)</td>
<td>Injection into adult ticks</td>
<td>Reduction in tick infection by <em>A. marginale</em></td>
<td>25, 42, 51</td>
</tr>
<tr>
<td><em>Haemaphysalis longicornis</em></td>
<td>Longicin</td>
<td>Microinjection into adult ticks</td>
<td>Inhibition of <em>B. gibsoni</em> killing by ticks</td>
<td>46</td>
</tr>
<tr>
<td><em>Dermacentor variabilis</em></td>
<td>GST</td>
<td>Injection into adult ticks</td>
<td>Reduction in <em>A. marginale</em> levels in tick guts after AF and salivary glands after TF</td>
<td>42</td>
</tr>
<tr>
<td><em>Dermacentor variabilis</em></td>
<td>Ubiquitin vATPase</td>
<td>Injection into adult ticks</td>
<td>Reduction in <em>A. marginale</em> levels in tick guts after AF</td>
<td>42</td>
</tr>
<tr>
<td><em>Dermacentor variabilis</em></td>
<td>M</td>
<td>Salivary selenoprotein</td>
<td>Injection into adult ticks</td>
<td>42</td>
</tr>
<tr>
<td><em>Ixodes scapularis</em> IDE8 cells</td>
<td>Proteasome</td>
<td>Incubation with dsRNA solution</td>
<td>Reduction in <em>A. marginale</em> levels</td>
<td>42</td>
</tr>
<tr>
<td><em>Ixodes scapularis</em> IDE8 cells</td>
<td>Ferritin</td>
<td>Incubation with dsRNA solution</td>
<td>Reduction in <em>A. marginale</em> levels</td>
<td>42, 51</td>
</tr>
<tr>
<td><em>Ixodes scapularis</em> IDE8 cells</td>
<td>Subolesin (4D8)</td>
<td>Incubation with dsRNA solution</td>
<td>Reduction in <em>A. marginale</em> levels</td>
<td>42, 51</td>
</tr>
<tr>
<td><em>Ixodes scapularis</em> IDE8 cells</td>
<td>Salp14</td>
<td>Incubation with dsRNA solution</td>
<td>Increase in <em>A. marginale</em> levels</td>
<td>42</td>
</tr>
</tbody>
</table>

Data updated after (15). Abbreviations: TROSPA, tick receptor for *B. burgdorferi* OspA; Salp, salivary gland protein; Isac, *I. scapularis* anticomplement; GST, glutathione-S transferase; AF, acquisition feeding; TF, transmission feeding; HSC, hematopoietic stem/progenitor cells protein-like.
These studies demonstrated a role of subolesin in Anaplasma-tick interactions, suggesting that this protein may be useful in a vaccine formulation for the control of tick infestations and pathogen infection/multiplication in ticks.

Recently, a functional genomics approach to characterize tick genes regulated in response to A. marginale infection in cultured IDE8 tick cells and ticks was reported by de la Fuente et al. (42). RNAi in D. variabilis ticks and IDE8 tick cells was used for functional studies of differentially expressed genes/proteins that were identified by suppression-subtractive hybridization and differential in-gel electrophoresis analyses. Through these studies molecules were identified that affected A. marginale infection in IDE8 tick cells and at different sites of development in ticks (Table 1). Importantly, the results revealed that a molecular mechanism occurs by which tick gene expression mediates the A. marginale developmental cycle and trafficking through ticks.

Collectively, these studies demonstrated that RNAi constitutes an important tool for the study of the tick-pathogen interface which will likely contribute to the rapid identification and characterization of candidate antigens for use in pathogen transmission-blocking tick vaccines.

4.3. Other potential approaches for the genetic manipulation of ticks and the characterization of the tick-pathogen interface

Recombinant bacteria, parasites and viruses that naturally infect ticks may be useful for developing novel methods for the study of tick-pathogen interactions and the manipulation of the tick-pathogen interface. However, the genetic transformation of intracellular bacteria that infect ticks has been difficult to attain because of their intracellular location, small size and complex growth conditions. Transformation and tick infection with recombinant bacteria has recently been reported for Escherichia coli (52, 53), B. burgdorferi (54-57), B. afzelii (58), and R. monacensis (59). Methods have been developed for the production and detection of recombinant organisms in cultured cells for the tick-borne rickettsial pathogens, R. prowazekii (60-62), C. burnetti (63, 64), A. phagocytophilum (65), Ehrlichia muris (66), and F. tularensis (67-69). Transformation of the protozoan parasite, B. bovis, with potential applications for other Babesia species, has also been reported (70).
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Therefore, paratransgenesis is an alternative approach for the manipulation of ticks by production of infective recombinant tick symbionts such as *Wolbachia* and other related organisms (71-73). Although these experiments have not been reported in ticks, recent developments suggest that transformation of *Wolbachia* may be feasible (74, 75). Additionally, the sequencing of the *I. scapularis* genome has revealed the presence of sequences with identity to *Rickettsia* spp., which are possible tick symbionts with a long co-evolutionary history (90).

These preliminary studies suggest the possibility of developing paratransgenic ticks with transformed pathogens for study of tick-pathogen interactions by expression of recombinant proteins and other molecules in the tick. Use of fluorescent bacteria and parasites may allow researchers to document pathogen infection, multiplication and trafficking in ticks (57). Antibiotic-resistant bacteria are an important tool for in vitro studies in cell culture. Recombinant bacteria and parasites could serve as a vehicle for delivery of recombinant proteins and dsRNAs for use in functional studies of tick biology and tick-pathogen interactions.

The infection of tick cells with vector-borne RNA viruses such as Semliki Forest virus was found to trigger the RNAi pathway and may be useful in characterizing dsRNA-mediated gene silencing in ticks (76). Recombinant viruses engineered to produce dsRNA may allow for induced and/or enhanced RNAi in vivo in ticks. This approach has been used to render mosquitoes resistant to arboviruses (77-80) and *Plasmodium* parasites (81), thus confirming the potential of this approach for targeting the vector-pathogen interface to control the transmission of vector-borne pathogens.

Finally, although remotely possible at this time, the production of transgenic ticks through embryo microinjection, electroporation or transfection may be feasible. While transformation methods with transposable elements similar to those developed for *Drosophila* (82) and mosquitoes (83) have not been established in ticks, electroporation and transfection of foreign DNA has been accomplished in shrimp embryos (84-86). Recently, Sunter *et al.* (91) discovered a short interspersed repetitive element (SINE; designed Ruka) that may be mobile in the genome of *R. appendiculatus*, *B. microplus*, *A. variegatum* and *I. scapularis*. Although at its infancy, this and other similar sequences present in tick genomes may be used to develop transformation methods in ticks. Transgenesis is being investigated as a means of engineering mosquitoes with a genetic trait that confers resistance to malaria or causes population suppression while driving the new trait through field populations (87). Recently, a promising result was reported in which transient sterile ticks were produced by RNAi of subolesin and may be used in the future development of methods for autocidal control of ticks (48). Development of techniques for production of transgenic ticks for autocidal control and resistance to pathogen transmission would enhance research in this area.

5. SUMMARY AND PERSPECTIVE

Tick vaccines and RNAi have proven useful for studying tick-pathogen interactions and for targeting the tick-pathogen interface with the goal of interrupting the transmission of tick-borne pathogens. Advances in the use and applications of RNAi in ticks, as well as development of new approaches for the genetic manipulation of ticks, will greatly enhance future opportunities to study the tick-pathogen interface. The combination of tick and pathogen genomics (88-90) with high throughput screening platforms using RNAi (92) and other forthcoming technologies will increase our knowledge of the tick-pathogen interface and will likely provide novel and improved vaccines targeted both at control of tick infestations and the transmission of tick-borne pathogens.

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**Abbreviations:** RNAi, RNA interference; TBEV, tick-borne encephalitis virus; siRNA, small interfering RNA; dsRNA, double-stranded RNA; Ago, Argonaute; PAZ, Piwi/Argonaute/Zwille; DEAD, i.e. the single letter code for amino acids Asp-Glu-Ala-Asp; RISC, RNA-induced silencing complex; TROSPA, tick receptor for *B. burgdorferi* OspA; Salp, salivary gland protein; Isac, *I. scapularis* anticomplement; GST, glutathione-S transferase; AF, acquisition feeding; TF, transmission feeding; HSC, hematopoietic stem/progenitor cells protein-like.

**Key Words:** Tick, Vaccine, RNA interference, Tick-pathogen interface, Evolution, Control, Paratransgenesis, Rickettsia, Anaplasma, Borrelia, Ehrlichia, Wolbachia, Virus, Review

**Send correspondence to:** Jose de la Fuente, Department of Veterinary Pathobiology, Center for Veterinary Health Sciences, 250 McElroy Hall, Oklahoma State University, Stillwater, OK 74078. Tel: 405-744-0372. Fax: 405-744-5275, E-mail: jose.de_la_fuente@okstate.edu

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