Identification of Rhipicephalus microplus Genes That Modulate the Infection Rate of the Rickettsia Anaplasma marginale

Ricardo F. Mercado-Curiel*, María L. Ávila-Ramírez*, Guy H. Palmer, Kelly A. Brayton*

Program in Vector-Borne Diseases, Department of Veterinary Microbiology and Pathology and Paul G. Allen School for Global Animal Health, Washington State University, Pullman, Washington, United States of America

Abstract

Arthropod vectors transmit a diversity of animal and human pathogens, ranging from RNA viruses to protozoal parasites. Chemotherapeutic control of pathogens has classically focused either on insecticides that kill the vector itself or antimicrobials for infected patients. The limitation of the former is that it targets both infected and uninfected vectors and selects for resistant populations while the latter requires prompt and accurate diagnosis. An alternative strategy is to target vector molecules that permit the pathogen to establish itself, replicate, and/or develop within the vector. Using the rickettsial pathogen Anaplasma marginale and its tropical tick vector, Rhipicephalus microplus, as a model, we tested whether silencing specific gene targets would affect tick infection rates (the % of fed ticks that are infected with the pathogen) and pathogen levels within infected ticks. Silencing of three R. microplus genes, CK187220, CV437619 and TC18492, significantly decreased the A. marginale infection rate in salivary glands, whereas gene silencing of TC22382, TC17129 and TC16059 significantly increased the infection rate in salivary glands. However in all cases of significant difference in the infection rate, the pathogen levels in the ticks that did become infected, were not significantly different. These results are consistent with the targeted genes affecting the pathogen at early steps in infection of the vector rather than in replication efficiency. Identifying vector genes and subsequent determination of the encoded functions are initial steps in discovery of new targets for inhibiting pathogen development and subsequent transmission.

Introduction

Arthropod vectors transmit a diversity of animal and human pathogens, ranging from RNA viruses to protozoal parasites. Chemotherapeutic control of pathogens has classically focused either on insecticides that kill the vector itself or antimicrobials for infected patients. The limitation of the former is that it targets both infected and uninfected vectors and thus broadly selects for resistant populations while the latter requires prompt and accurate diagnosis. An alternative strategy is to target vector molecules that permit the pathogen to establish itself, replicate, and/or develop within the vector, thus specifically targeting only the small proportion of infected vectors.

Vector competence, the ability to acquire and transmit pathogens, is a multifactorial process and involves multiple genes and gene networks in multiple organs. The vector midgut and salivary glands are attractive targets as these organs represent, respectively, sites of initial colonization and secretion into the saliva for transmission [1,2,3,4,5]. Using the rickettsial pathogen Anaplasma marginale and its tropical tick vector, Rhipicephalus microplus, as a model, we previously identified a set of tick midgut and salivary gland genes that are regulated in response to pathogen infection [5]. We supplemented this set with R. microplus genes for which the expressed protein has been shown to vary in response to babesial infection [6,7]. Six candidate genes were selected based on bioinformatics analysis and an initial screen using post-transcriptional gene silencing by small interfering RNA (siRNA) (Table 1). Silencing of these six genes was then used to test two related hypotheses in the A. marginale/R. microplus model. The first was that silencing of the selected R. microplus genes affects the A. marginale infection rate (the % of fed ticks that acquire infection) in the tick midguts or salivary glands. The second hypothesis was that silencing of the selected R. microplus genes affects the level of A. marginale within infected ticks. Herein, we present the results of these experiments and discuss the findings in the context of the interface between tick biology and pathogen transmission.

Materials and Methods

Experimental Animals and Ticks

Animals were maintained according to IACUC protocol #2010-54 authorized by the University of Idaho Institutional...
Table 1. Bioinformatic analysis of candidate genes.

| Annotation (putative) | Best alignment | Species | E value | 5' end | TM | SP | CDD |
|-----------------------|----------------|---------|---------|--------|-----|----|-----|
| Secreted protein      | TC18492        | Is      | 3e-67   | Y      | –   | -  | CD07599 |
| Aldehyde dehydrogenase| TC16059        | Is      | 0       | N      | Y   | N  | CD07141 |
| Glutamine synthetase  | TC17129        | Tc      | 5e-133  | N      | N   | N  | PLN03036 |
| NADH-ubiquinone reductase| TC22382     | Av      | 6e-146  | N      | N   | N  | pfam10588,pfam13510 |
| Tat binding protein 1-interacting protein | CK187220 | Is      | 1e-86   | Y      | N   | N  | PHA02592 |

- Reports the GenBank accession number of the sequence with the lowest E value.
- Indicates whether the alignment with a known protein indicates the presence of the first coding amino acid in the cDNA sequence; Y = yes; N = no.
- Indicates whether there are any recognized transmembrane domains using TMpred; Y = yes; N = no.
- Indicates the presence of a signal peptide; Y = yes; N = no. Note: if No is indicated in the 5' end column, the positive SP prediction may actually reflect the presence of a TM domain near the start of the sequence rather than a true signal peptide.

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Small Interfering RNA

Two different double-stranded siRNAs were specifically designed and chemically synthesized for each selected gene (Integrated DNA Technologies, Inc). Synthetic short RNA duplexes had a 2-base 3'-overhang on the antisense strand, and were blunt on the other end; the 3' end of the sense strand contained two DNA instead of RNA bases. The two siRNA duplexes designed for each selected gene are listed in Table 2. The siRNAs were suspended in Nuclease Free Duplex Buffer (Integrated DNA Technologies, Inc).

Labeling and Injection of Ticks with siRNA

Freshly molted male ticks were allocated to specific treatment groups and ticks within a group identified by removal of a single leg between the third and fourth segment. Ticks were injected with 0.5 μl of a 10 pmol/μl stock solution of one of the specific siRNA duplexes described above. Control groups were injected with an equivalent volume of Nuclease Free Duplex Buffer (Integrated DNA Technologies, Inc). The injection was performed using a 10 μl syringe (Hamilton) with a borosilicate glass needle coupled to a 33 gauge 15 mm metal needle (Hamilton), and the desired administered volume was controlled by the UMP3 Microsyringe Injector and Micro4 Controller (World Precision Instruments).

The glass needles were made from borosilicate glass capillaries (Harvard Apparatus) using a P2000 laser-based micropipette puller (Sutter Instrument Co.). The injection procedure was carried out at the base of the 4th leg through the scleritized coxal membrane. No reflux of the injected solution, hemolymph or tissue was observed from the site of the puncture when the glass needle was carefully withdrawn.

Approximately 5 hrs following the corresponding procedure, labeled/injected tick groups were allowed to acquisition feed on the A. marginale infected calf during acute bacteremia. Ticks were allowed to feed for 6 days and then removed and individually dissected for collection of salivary glands or midguts within 48 hrs. One half of the tissue was put in Trizol (Invitrogen), and the other half in Cell Lysis Buffer (Qiagen) containing 2 mg/ml proteinase K (Invitrogen), and stored at −70°C until total RNA or genomic DNA extractions were performed for gene silencing, or infection level/rate and β-actin level determinations, respectively.

Gene Silencing, A. marginale Infection and R. microplus Actin Determination in Single Tick Tissues

In order to assess the gene silencing effect, total RNA extracted from dissected tissues, either half of the midgut or one salivary gland, was treated with DNase (Applied Biosystems). Random primed, single stranded cDNA was synthesized using the SuperScript III First-Strand Synthesis SuperMix for qRT-PCR kit (Invitrogen), and analyzed by TaqMan quantitative PCR.

Animal Care and Use Committee in accordance with institutional guidelines based on the U.S. National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. Two Holstein calves, 4 months of age (#C36185, #C36190), were used in this study. These animals had no previous exposure to ticks. One animal was inoculated intravenously with approximately 10⁹ A. marginale (St. Maries strain). The second uninfected calf was used for rearing 4 grams, approximately 80,000 larvae, of *Rhipicephalus microplus* ticks (La Minita stock) to the engorged nymph stage. Molting nymphs were manually collected from the calf after 14 days, and incubated at 26°C, 95% humidity to complete molting to the adult stage. Unfed adult ticks were sorted by sex and the males used for silencing of selected genes within 36 hrs of molting.
(qPCR), using iQ-Supermix reagents (BioRad, Hercules, CA), to determine the gene expression level of each siRNA-target gene; R. microplus actin was used for normalization. All Taqman qPCR assays were carried out using aliquots from the same cDNA sample. Control reactions without reverse transcriptase or cDNA were carried out to confirm the absence of DNA contamination in the RNA samples or contamination in the qPCR reaction, respectively. The sets of primers and TaqMan probes used for each analyzed gene are listed in Table 2. All assays were done in triplicate.

Genomic DNA extracted from dissected tissues, either half of the midgut or one salivary gland, was analyzed by qPCR for the single copy gene msp5 to determine the A. marginale infection level and R. microplus actin level as previously described [5,8]. qPCR assays were carried out in triplicate for the unknown DNA samples, simultaneously with serial dilutions of cloned msp5 or actin, using the iQ-Supermix (BioRad, Hercules, CA). The primers and TaqMan probes used are listed in Table 2. The number of positive samples that showed a quantifiable amount of A. marginale was used to determine the infection rate, dividing it by the total number of samples in the corresponding group of singly collected tick tissues.

### Statistical Analysis

Statistical analysis of gene silencing data (gene copies/10^3 β-actin), infection level (mean no. bacteria/total organ), and R. microplus actin level in individual organ (β-actin gene copies ×10^3/organ) were performed using Minitab 15 version 1.30.0; consisted of One-way analysis of variance (ANOVA) with a factor of three and six, sixteen, and sixteen levels, respectively, and Tukey’s family error rate of 5; p values of less than 0.05 were considered statistically significant.

### Results

#### Selection of Genes

Using a gene expression microarray we identified several R. microplus genes whose expression is regulated upon infection with A. marginale [5]. We supplemented this set with R. microplus genes encoding proteins that were described as being regulated upon Babesia bovis infection, a protozoal pathogen that uses the same tick...
vector [6,7]. We selected six genes from these sets using a combination of results from a pilot silencing experiment and bioinformatics analysis (Table 1; see http://compbio.dfci.harvard.edu/cgi-bin/tgi/gbrowse/bmiG12.1 for all listings of genes). TC10492 showed one of the highest fold changes in transcription in A. marginale infected tick salivary glands, 3.7 and 6.5 at 6 and 9 days, respectively, which was congruent with the protein overexpression in the tick upon B. bovis infection [5,6]. TC16059 and TC17129 showed increased protein expression upon B. bovis infection, respectively, while mRNA levels remained unaffected upon A. marginale infection. CK187220 was transcriptionally down-regulated during early infection and, subsequently, in the salivary gland. As a control, CV437619 was selected as a control as there was no evidence of regulation upon pathogen infection in the tick salivary gland [5,6,7]. These five genes were examined in the salivary gland, which is the relevant tissue in which A. marginale undergoes final replication prior to transmission. TC22382 was examined in both the tick midgut and the salivary gland based on the two factors: i) bioinformatic analysis that suggested a possible role of this gene in electron and proton transport that may affect specific midgut physiological processes like uptake of blood meal components, diuresis, and water balance (Table 1); and ii) discrepant results in the midgut following infection with the two different pathogens: increased protein expression in the midgut upon B. bovis infection but a 5-fold decrease in mRNA levels with A. marginale infection.

Tick Survival

Because injection of adult male B. microplus ticks had not been previously reported, we first determined the survival rates for this procedure as well as for the procedure used to identify ticks by treatment group, removal of one of the eight legs. Tick survival was evaluated as the proportion of treated ticks that were recovered alive after 20 days of feeding (6 days after Nuclease Free Duplex Buffer injection and/or clipping of one leg for identification). Ticks subjected to both injection and clipping of a leg had a much lower survival (27%); this data was used to determine the group size to be used for injection of siRNA.

Gene Silencing with Two Different Specific siRNAs

Evidence for off-target effects of siRNA in arthropod systems has been reported [9,10,11,12]. In an effort to control for off-target effects, two different double-stranded siRNAs were specifically designed for each gene and are referred to as (gene identifier) siRNA_A and (gene identifier) siRNA_B. The possibility of having equivalent specific and off-target effects with the use of two different siRNAs is low, and provide better support that the resulting phenotype is due to a specific inhibition of the cognate mRNA. The effect of siRNAi is systemic with gene silencing effects occurring throughout the whole tick [13,14]. RNA extracted from individual salivary glands or from half a midgut was analyzed by qRT-PCR to determine the gene silencing effect (Figure 1).

Injection with CK187220 siRNA_A and CK187220 siRNA_B resulted in a statistically significant silencing effect of 81% and 84%, respectively, in salivary glands. There was no significant difference in the silencing effects of the two siRNAs (Figure 1). Treatment with CV437619 siRNA_A and CV437619 siRNA_B resulted in salivary gland expression levels of CV437619 that were not significantly different as compared to the controls (Figure 1). This may be due to the low expression levels of CV437619 in the controls, making it more difficult to detect a significant reduction following siRNA treatment. TC18492 siRNA_A and TC18492 siRNA_B caused a statistically significant silencing effect of 93% and 80%, respectively, in salivary glands. There was no significant difference between the effects of the two siRNAs (Figure 1). The silencing effects of TC17129 siRNA_A and TC17129 siRNA_B in salivary glands were 90% and 73%, respectively, which were statistically significantly different both one from another and as compared to the control group (Figure 1). Both TC16059 siRNA_A and TC16059 siRNA_B caused a statistically significant silencing effect of 47% and 61% in salivary glands, respectively; there was no significant difference between the effects of both siRNAs (Figure 1).

The silencing effect of TC22382 siRNA_A and TC22382 siRNA_B were investigated in the midgut as well as in the salivary glands due to the potential role of this transporter to affect midgut physiology involving uptake of bloodmeal components, diuresis, and water balance. In the midgut TC22382 siRNA_A and TC22382 siRNA_B caused a statistically significant silencing effect of 43% and 57%, respectively; there was no significant difference between the effects of the two siRNAs (Figure 1). In salivary glands, the reduction was 20% and 40%, respectively (Figure 1). Only the TC22382 siRNA_B group was significantly different as compared to the control group.

Effect of Gene Silencing on A. marginale Infection Rate and Level

During acquisition feeding, the ticks were exposed to A. marginale levels ranging from 6 × 10^7–8.5 × 10^8 organisms/ml of blood. Control ticks had infection rates (% of fed ticks that acquired infection) of 100% and 60% in midgut and salivary glands, respectively (Table 3). Silencing with both members of each set of gene-specific siRNAs, siRNA_A and siRNA_B, showed the same outcome in all cases. All three possible outcomes were observed with one or more of the gene-specific siRNA sets: an increase, decrease or no effect on the infection rate (Table 3). Gene silencing of CK187220, CV437619, and TC18492 resulted in statistically significant decreases in the salivary gland infection rate, whereas gene silencing of TC17129 and TC16059 significantly increased the infection rate. Silencing of TC22382 in the salivary gland also resulted in a statistically significant increase in infection rate; however no increase was detectable in the midgut as the control ticks also had a 100% infection rate (Table 3). The corresponding infection level for each group reflects the mean infection level of samples within the group that showed a quantifiable amount of A. marginale (Table 3). The infection levels, reported as the mean number of organisms per salivary gland pair or midgut, were not statistically significantly different when comparing both gene-specific siRNA injected groups with each other or with their respective salivary gland or midgut control groups (Table 3).

Effect of Gene Silencing on Tissue Development/Maintenance

It has been reported that gene silencing affected tick organ development generating smaller or altered tissues [15,16,17]. To investigate if silencing of our selected genes had an effect on the midgut or salivary gland, the tissue actin levels in individual organs were determined by qPCR for all ticks from all groups using aliquots from the same DNA samples used to detect and measure A. marginale infection. All DNA samples showed detectable quantities of actin DNA (Figure 2). The amount of actin was statistically significantly lower (p<0.05) in salivary glands for groups injected with siRNAs for CK187220, CV437619, and TC18492. These groups also demonstrated lower A. marginale infection rates (Table 3). No statistically significant differences in actin levels were observed in midguts or salivary glands from groups injected with siRNAs corresponding to TC22382, TC17129 and
Correlation between *A. marginale* Infection and Actin Levels

Salivary glands from control ticks had actin levels that ranged from $4.0 \times 10^5$ to $3.5 \times 10^6$. In contrast, the levels were consistently lower for three siRNA groups: CK187220, CV437619 and TC18492 (Figure 3). However, the actin level appeared to be independent of the infection level exhibited by the individual ticks in both the siRNA injected and control groups, with $r$ values ranging from 0.05 to 0.69.

**Discussion**

In the present study we tested two linked hypotheses. The first hypothesis, silencing of *R. microplus* genes significantly affects the *A. marginale* infection rate in the tick, was accepted based on the observation that gene silencing resulted in a decrease (CK187220,
Table 3. *Anaplasma marginale* infection levels and rates following gene silencing.

| Infection Rate | Group | Injected With | Tissue Analyzed | Mean no. bacteria/total organ (±SD)b | Infection Level (No. positive/No. recovered) ×100 | Survival Rate (No.recovered/No. injected) ×100 |
|----------------|-------|---------------|-----------------|-------------------------------------|-----------------------------------------------|------------------------------------------------|
| Control        | Nuclease Free | SG       | 2.85×10^6 (±1.20) | 59.45 (22/37) | 13.21 (37/280) |
| Lower          | CK187220 Duplex Buffer | MG       | 1.00×10^6 (±0.43) | 100 (8/8) |
|                | CK187220 siRNA_A | SG       | 1.07×10^6 (±0.16) | 41.66 (15/36) | 20 (36/180) |
|                | CV437619 siRNA_A | SG       | 2.74×10^6 (±0.45) | 30.43 (7/23) | 12.77 (23/180) |
|                | CV437619 siRNA_B | SG       | 2.12×10^6 (±2.18) | 31.25 (15/48) | 26.66 (48/180) |
|                | CV437619 siRNA_A | SG       | 1.45×10^5 (±1.60) | 41.66 (10/24) | 13.33 (24/180) |
|                | TC18492 siRNA_A | SG       | 5.09×10^5 (±0.54) | 40 (10/25) | 12.5 (25/200) |
|                | TC18492 siRNA_B | SG       | 1.25×10^5 (±1.80) | 45.45 (15/33) | 16.5 (33/200) |
| Higher         | TC22382 siRNA_A | SG       | 4.05×10^6 (±0.35) | 100 (3/3) | 3 (3/100) |
|                | TC22382 siRNA_B | SG       | 8.10×10^6 (±0.54) | 100 (15/15) | 15 (15/100) |
|                | TC17129 siRNA_A | SG       | 8.86×10^6 (±0.97) | 77.79 (7/9) | 11.25 (9/80) |
|                | TC17129 siRNA_B | SG       | 1.41×10^7 (±1.14) | 82.60 (19/23) | 28.75 (23/80) |
|                | TC16059 siRNA_A | SG       | 1.18×10^7 (±2.22) | 87.5 (7/8) | 10 (8/80) |
| Equal          | TC16059 siRNA_B | SG       | 7.49×10^6 (±0.51) | 90 (18/20) | 25 (20/80) |
|                | TC22382 siRNA_A | MG       | 7.90×10^6 (±0.85) | 100 (3/3) | 3 (3/100) |
|                | TC22382 siRNA_B | MG       | 1.53×10^6 (±0.63) | 100 (15/15) | 15 (15/100) |

*SG = Salivary glands, MG = midguts.
*SD = Standard deviation.
*These values were not considered when calculating the mean rate since these midguts where dissected from the same ticks that were used for dissecting salivary glands for TC22382 siRNA_A and TC22382 siRNA_B analysis.

*Indicates that blocking TC18492 expression may be effective in blocking transmission of multiple pathogens.

TC18492, CV437619 or an increase (TC22382, TC17129, TC16059) in the proportion of exposed ticks that acquired salivary gland infection (Table 3). Molecules that inhibited CK187220, TC18492, CV437619 expression or function would be candidates for development of transmission blocking therapeutics. Although there are no significant homologs to CK187220 in the current data bases, the nucleotide sequence of TC18492 showed 85% identity with three *Dermacentor variabilis* contigs (GenBank accession no. EZ325453.1, EZ524522.1, EZ533121.1) identified in pooled RNA isolated from unfed, uninfected adult male *D. variabilis*, partially fed males naturally infected with *A. marginale*, and unfed adult females infected with bacteria and fungi [18]. The deduced amino acid sequence of TC18492 also revealed 56% identity (E value 2×10^-67) with an *Ixodes scapularis* putative secreted protein (GenBank accession no. XP_002409139.1). The TBP-1 superfamily has several tases from ticks (*Aedes aegypti, Anopheles gambiae*), partial-fed males naturally infected with *A. marginale*, but also affect normal cellular processes. Silencing of any one of CK187220, TC18492, and CV437619, decreased overall actin expression following siRNA treatment was measured by normalization against actin. However, the level of salivary gland actin itself was decreased following injection with these siRNAs (Figure 2), thus the expression of these three genes was even more profoundly reduced than indicated by normalization against actin levels (Figure 1). This is especially relevant in the case of CV437619 where the silencing effect was not statistically significant when normalized against actin but there was a significant decrease in *A. marginale* infection rates. CV437619 shares 73% identity (E value, 8×10^-67) with an *Ixodes scapularis* Tat binding protein 1 (TBP-1)-interacting protein (GenBank accession no. XP_002409139.1). The TBP-1 superfamily has several members that are components of the 26S proteasome, a basic multi-protein complex that degrades ubiquitinated proteins in an ATP-dependent fashion. That knockdown of all three genes, CK187220, TC18492, and CV437619, decreased overall actin levels suggests that the effects not only diminish pathogen infection but also affect normal cellular processes. Silencing of any one of the three genes did not completely abolish infection with *A. marginale*, however, suppression of gene combinations may have an additive or synergistic effect on blocking infection.

Silencing of TC22382, TC17129, and TC16059 resulted in increased *A. marginale* salivary gland infection rates. This suggests that the encoded proteins may normally function to inhibit infection, either directly or as members of cellular pathways. TC22382 has significant identity with NADH-ubiquinone reductase from ticks (*Amblyomma variegatum, Ixodes scapularis*), mosquitoes (*Aedes aegypti, Anopheles gambiae*) and flies (*Drosophila pseudoobscura*). NADH-ubiquinone reductase is a conserved metabolic enzyme located in the inner mitochondrial membrane that catalyzes the transfer of electrons from NADH to coenzyme Q (CoQ). Interestingly, TC22382 is up regulated approximately three-fold in the midgut of adult male *Dermacentor andersoni* upon feeding [5], consistent with increased energy needs upon uptake of blood meal components, diuresis, and water balance. In addition, innate immune responses also require increased energy, thus decreased expression may lower innate responses in the midgut epithelium. Unfortunately, the 100% infection rate in the midgut of controls
Figure 2. Effect of gene silencing on actin levels in tick organs. Interval plot of the distribution of *R. microplus* actin level in individual organs along the different gene-specific siRNA_A and siRNA_B injected groups, showing their central tendency and variability at a 95% confidence level. Asterisk (*) indicates statistically significant difference (p<0.05) when comparing to the respective control group.

Figure 3. Correlation between *A. marginale* infection and actin levels. Scatterplot assessing the relationship between the two variables: *A. marginale* infection and actin levels, in individual pair of salivary glands from control group, and the two gene specific siRNA_A and siRNA_B injected groups for CK187220, CV437619, and TC18492. Both infected and uninfected tissues were included in the analysis; values from uninfected salivary glands can be visualized on the X-axis.
mosquito species, including *R. microplus* ticks would be consistent with this role. Increased expression, suggested to be a protective mechanism against infection was associated with increased glutamine synthetase and ammonia [19]. In murine models of malaria and in *Schistosoma mansoni* infection of its molluscan host *Biomphalaria glabrata*, infection was associated with increased glutamine synthetase expression, suggested to be a protective mechanism against infection-induced increases in glutamate levels [20,21]. The increased *A. marginale* infection rates upon TC17129 silencing in *R. microplus* ticks would be consistent with this role.

TC16059 has identity to aldehyde dehydrogenase from ticks including *Ixodes scapularis* and *Amblyomma variegatum*, as well as several mosquito species, including *Culex quinquefasciatus*, *Aedes aegypti* and *Anopheles gambiae*. NAD\(^+\)-dependent enzymes in the aldehyde dehydrogenase superfamily are, in general, oxidoreductases that oxidize a wide range of endogenous and exogenous aliphatic and aromatic aldehydes, playing an important role in aldehyde detoxification. Additionally, they participate in 17 metabolic pathways such as glycolysis, gluconeogenesis, fatty acid and pyruvate metabolism, and pentose and gluconuronate interconversions, and serve as binding proteins and osmoregulants. Aldehyde dehydrogenase is stress-induced and glucose-repressed, and has been shown to play a role in insecticide resistance in *Culex quinquefasciatus* [22]. TC16059 and other aldehyde dehydrogenases share a number of highly conserved residues necessary for catalysis and cofactor binding. TC16059 may have an infection-derived stress protective function against *A. marginale* infection, which would explain the increased infection rate (Table 3) associated with its silencing.

The second hypothesis, that silencing of the selected *R. microplus* genes affects the level of *A. marginale* infection within infected ticks, was rejected. This suggests that the targeted genes influence the pathogen at early steps in infection of the vector rather than in replication once infection is established. Importantly, the number of infected ticks has been shown to be a determinant of whether onward transmission to new mammalian hosts is successful, thus decreasing the infection rate, even if independent of the infection level, is likely to be successful in blocking transmission [Ueti et al., 2007, 2009; Agnes et al., 2010; Herndon et al, 2013]. Although there was variation in survival rates within and among treatment groups, these were not significantly different from the survival rate of the control group (Table 3). This is consistent with tick death being a consequence of the infection procedures rather than a specific effect of the siRNA. This interpretation is also supported by differential survival rates between tick cohorts injected with two different siRNAs targeting the same gene as a gene specific effect on tick survival would be expected to be similar between the siRNA_A and B treatments. Nonetheless, the possibility of gene specific effects on tick growth and survival cannot be definitively excluded and can be addressed by including data such as engorgement weight to better assess growth and development. Refined of the siRNA injection protocol to markedly improve survival in the control groups would facilitate detection of effects on tick growth and development and allow these to be discriminated from effects limited to pathogen entry and survival.

**Conclusions**

Silencing of three *Rhipicephalus microplus* genes, CK187220, CV437619 and TC184992, significantly decreased the *Anaplasma marginale* infection rate in salivary glands, whereas gene silencing of TC22382, TC17129 and TC16059 significantly increased the infection rate in salivary glands. However in all cases of significant difference in the infection rate, the pathogen levels in the ticks that did become infected, were not significantly different. These results indicate that the targeted genes influence the pathogen at early steps in infection of the vector and provide specific targets for further testing that could lead to the development of small molecule inhibitors as transmission blocking chemotherapeutics.

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**Author Contributions**

Conceived and designed the experiments: RFMC GHP KAB. Performed the experiments: RFMC MLAR KAB. Analyzed the data: RFMC MLAR. Contributed reagents/materials/analysis tools: RFMC MLAR GHP KAB. Performed the experiments: RFMC MLAR KAB. Analyzed the data: RFMC MLAR. Contributed reagents/materials/analysis tools: RFMC MLAR GHP KAB. Wrote the paper: RFMC GHP KAB.

**References**

1. Karim S, Singh P, Ribeiro JM (2011) A deep insight into the sialotranscriptome of the gulf coast tick, *Amblyomma maculatum*. PLoS ONE 6: e20352.
2. Francischetti IM, Anderson JM, Manouskas N, Pham VM, Ribeiro JM (2011) An insight into the sialotranscriptome and proteome of the coarse bovengalled tick, *Hyalomma marginatum* rufipes. J Proteomics 74: 2892–2898.
3. Francischetti IM, My Pham V, Mans BJ, Andersen JF, Mathur TN, et al. (2005) The transcriptome of the salivary glands of the female western black-legged tick *Ixodes pacificus* (Aves. Ixodidae). Insect Biochem Mol Biol 35: 1142–1161.
4. Ribeiro JM, Anderson JM, Manouskas NC, Meng Z, Francischetti IM (2011) A further insight into the sialome of the tropical bont tick, *Amblyomma variegatum*. BMC Genomics 12: 136.
5. Mercado-Curiel RF, Palmer GH, Guererro FD, Brayton KA (2011) Temporal characterisation of the organ-specific *Rhipicephalus microplus* transcriptional response to *Anaplasma marginale* infection. Int J Parasitol 41: 851–860.
6. Rachinsky A, Guerrero FD, Scoles GA (2008) Proteomic profiling of *Rhipicephalus (Boophilus) microplus* midgut responses to infection with *Babesia bovis*. Vet Parasitol 152: 294–313.
7. Rachinsky A, Guerrero FD, Scoles GA (2007) Differential protein expression in ovaries of uninfected and *Babesia*-infected southern cattle ticks, *Rhipicephalus (Boophilus) microplus*. Insect Biochem Mol Biol 37: 1291–1308.
8. Scoles GA, Ueti MP, Nob SM, Kozoeles DP, Palmer GH (2007) Conservation of transmission phenotype of *Anaplasma marginale* (Rickettsiales: Anaplasmataceae) strains among *Demodex* and *Rhipicephalus* ticks (*Acari: Ixodidae*). J Med Entomol 44: 484–491.
9. Ma Y, Creanga A, Lunn L, Beachy PA (2006) Prevalence of off-target effects in *Drosophila* RNA interference screens. Nature 443: 359–363.
10. Cullen BR (2006) Enhancing and confirming the specificity of RNAi experiments. Nat Methods 3: 677–681.
11. Rual JF, Kinross N, Achat G (2007) Novel insights into RNAi off-target effects using *C. elegans* paradigms. BMC Genomics 8: 106.
12. Lew-Tabor AE, Kurscheid S, Barrero R, Gondro C, Moolhuijzen PM, et al. (2007) A deep insight into the sialotranscriptome of the gulf coast tick, *Amblyomma maculatum*. PLoS ONE 6: e20352.
Karim S, Miller NJ, Valenzuela J, Sauer JR, Mather TN (2005) RNAi-mediated gene silencing to assess the role of synaptobrevin and cystatin in tick blood feeding. Biochem Biophys Res Commun 334: 1336–1342.

Soares CA, Lima CM, Delan MC, Piersman J, Beard CB, et al. (2005) Capillary feeding of specific dsRNA induces silencing of the isac gene in nymphal Ixodes scapularis ticks. Insect Mol Biol 14: 443–452.

Mulenga A, Khumthong R (2010) Disrupting the Amblyomma americanum (L.) CD147 receptor homolog prevents ticks from feeding to repletion and blocks spontaneous detachment of ticks from their host. Insect Biochem Mol Biol 40: 524–532.

Nijhof AM, Taoufik A, de la Fuente J, Kocan KM, de Vries E, et al. (2007) Gene silencing of the tick protective antigens, Bm86, Bm91 and subolesin, in the one-host tick Boophilus microplus by RNA interference. Int J Parasitol 37: 653–662.

de la Fuente J, Almazan C, Blas-Machado U, Naranjo V, Mangold AJ, et al. (2006) The tick protective antigen, 4D8, is a conserved protein involved in modulation of tick blood ingestion and reproduction. Vaccine 24: 4082–4095.