Video Article

RNA Interference in Ticks

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Abstract

Ticks are obligate hematophagous ectoparasites of wild and domestic animals and humans, and are considered to be second worldwide to mosquitoes as vectors of human diseases[1] and the most important vectors affecting cattle industry worldwide[2]. Ticks are classified in the subclass Acari, order Parasitiformes, suborder Ixodida and are distributed worldwide from Arctic to tropical regions[3]. Despite efforts to control tick infestations, these ectoparasites remain a serious problem for human and animal health[4,5].

RNA interference (RNAi)[6] is a nucleic acid-based reverse genetic approach that involves disruption of gene expression in order to determine gene function or its effect on a metabolic pathway. 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[7-9]. Post-transcriptional gene silencing mechanisms initiated by dsRNA have been discovered in all eukaryotes studied thus far, and RNAi has been rapidly developed in a variety of organisms as a tool for functional genomics studies and other applications[10].

RNAi has become the most widely used gene-silencing technique in ticks and other organisms where alternative approaches for genetic manipulation are not available or are unreliable[5,11]. The genetic characterization of ticks has been limited until the recent application of RNAi[12,13]. In the short time that RNAi has been available, it has proved to be a valuable tool for studying tick gene function, the characterization of the tick-pathogen interface and the screening and characterization of tick protective antigens[14]. Herein, a method for RNAi through injection of dsRNA into unfed ticks is described. It is likely that the knowledge gained from this experimental approach will contribute markedly to the understanding of basic biological systems and the development of vaccines to control tick infestations and prevent transmission of tick-borne pathogens[15-19].

Video Link

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Protocol

1. Generation of dsRNA.

1. Synthesize oligonucleotide primers containing T7 promoter sequences for *in vitro* transcription and synthesis of dsRNA (for example, for *Dermacentor variabilis* subolesin use oligonucleotide primers D8AAT75: 5'-TAATACGACTCACTATAGGGTACTGACTGGGATCCCCTGCACAGT-3' and D8DVT73: 5'-TAATACGACTCACTATAGGGTACTCGAGCTTGGTGGAAAGGACG-3').
2. Amplify target gene by RT-PCR using 10 pmol of each oligonucleotide primer and 1-10 ng of tick total RNA.
3. Purify the PCR product.
4. Synthesize dsRNA using 8 µL of the purified PCR product.
5. Quantify dsRNA by spectrometry.

2. Injection of Ticks with dsRNA.

2.1. Preparation of ticks for injection.

1. First, wash the ticks in a series of solutions by shaking them in each solution in a 50 mL disposable centrifuge tube, decanting the solution through a fine mesh wire screen over the tube top to retain the ticks. The sequence of the solutions for washing ticks is tap water, 3% hydrogen peroxide, two washes of distilled water, 70% ethanol and two more washes with distilled water.
2. Blot-dry the ticks on paper towels.
3. Count the ticks into groups of 20 to 50, depending upon the experiment, place the ticks from each group in a 1.25 oz plastic cup with a tightly fitted lid and label with the experimental group number.

2.2. Tick injection team.

The RNAi team consists of three people: (1) one person who positions each tick on double sticky tape affixed to a sheet of red dental wax, (2) one person who injects the ticks and (3) one person who monitors the ticks after injection, breathes CO_2 on the ticks to activate them and counts the living ticks into cups labeled with the experimental group number. All team members must wear disposable gloves.

2.3. Placement of ticks for injection.

1. Capture a tick using Dumont fine forceps and place it ventral side up on double sticky tape affixed to a 3" x 6" sheet of red dental wax. The ticks are closely positioned together in groups of 5 ticks.
2. Place a small strip of masking tape over the mouthparts of all 5 ticks in order to further restrain them but while leaving most of the body exposed so that the injection process can be observed by the tick injector (Figure 1).

2.4. Injection of ticks.

1. The ticks will be injected in the lower right quadrant of the ventral surface of the exoskeleton.
2. First, pierce a hole in the exoskeleton using a Monoject insulin syringe fitted with a ½", 29 gauge needle (Figure 2a).
3. Inject ticks immediately with 0.2-0.5 µL of dsRNA solution (5×10^{10} - 5×10^{11} molecules per µL) using a custom-made Hamilton syringe with a 1 inch, 33 gauge needle with a 45° beveled point (Figure 2b). The needle should be placed well into the tick cavity to insure the placement and retention of the dsRNA. Some fluid is likely to escape from the injection site (Figure 2c). Care should be taken not to over inject the ticks, which would cause loss of hemolymph and could cause the death of the tick.
4. Clean the Hamilton syringe after completing the injections in each experimental group. before using for another experimental group. Fill the syringe first from a beaker containing 3% hydrogen peroxide and then expel into a waste container, and repeat 15 times. Fill the syringe from a beaker containing sterile water and then expel into a waste container, and repeat 15 times. Take care not to bend the plunger of the Hamilton syringe because, if bent, the plunger will not move smoothly and respond to the gentle touch required for injection of ticks.

2.5. Treatment of ticks after injection.

1. Pick up the injected tick immediately from the double sticky tape with the fine forceps and place it in a plastic recovery container (approximately 6" x 6" and ringed with masking tape to prevent escape of the ticks). The ticks will be briefly inactive after injection but should soon begin to crawl around the dish.
2. Breath CO_2 onto the ticks immediately after placing them in the recovery container to help activate the ticks. Once the ticks are crawling and active, the injection wound will heal rapidly and they will most likely survive.
3. Count the ticks according to the number in each experimental group and place them in a labeled plastic cup with a tightly fitted lid. Replacement ticks should be injected to replace any that any died before injecting the next experimental group.

2.6. Tick holding.

1. Place the ticks in a humidity chamber (12hr light: 12 hr dark photoperiod at 22-25°C and 95% relative humidity) and hold for 1 day.
2. Place ticks in tick-feeding cells, one per experimental group, glued to a sheep and allow them to feed with an equal number of uninjected male or female ticks (whichever sex was not injected). Female ticks that feed to repletion, those that are removed from the sheep after 10 days of feeding or when the control females have dropped off the host are collected and weighed.
3. Place the ticks in cartons, and hold in the humidity chamber until completion of oviposition. Evaluate oviposition by weighting the egg mass produced by all ticks in the group.

2.7. Analysis of tick phenotype after RNAi.

1. Evaluate tick phenotype after feeding by determining the number of ticks that survived, tick weight, oviposition and egg fertility. However, other analyses may be performed depending on the targeted gene and objectives of the study.

3. Analysis to Confirm Gene Silencing by RT-PCR.

1. Dissect salivary glands and guts from individual ticks from control-injected and dsRNA-injected groups after feeding.
2. Extract total RNA from individual tissue samples.
3. Analyze target gene transcripts in individual tissues by real-time RT-PCR and normalize RNA levels against tick 16S rRNA using the genNorm method (ddCT method as implemented by Bio-Rad iQ5 Standard Edition, Version 2.0).
4. Run dissociation curves at the end of the reaction to ensure that only one amplicon is formed and that the amplicons denature consistently in the same temperature range for every sample.
5. Compare mRNA levels (normalized Ct values) between control-injected and dsRNA-injected ticks using the Student`s t-test (P=0.05).

4. Representative Results:

The protocol described herein has been used in our laboratory for RNAi in many different ixodid tick species (Table 1). The amount of dsRNA injected into the ticks varies with the size of the tick; larger tick species can accommodate a larger volume. Negative control ticks should be injected with an unrelated dsRNA. Several dsRNAs such as subolesin[14-19,22-25,27-32,34] and beta-actin[20,21] could be used as positive controls. Note that it is important to wash the syringe between treatments to avoid mixing dsRNA solutions. If the protocol is done correctly, less than 5% mortality should be obtained from the injection procedure after 24 hours. A typical phenotype after gene knockdown in ticks is shown in Figure 3 with a panel of ticks injected with pools of dsRNA in order to screen for tick protective antigens.

Tick species	dsRNA injected	References
Ixodes scapularis	cDNA library, subolesin, actin, nucleotidase, NF-kB, akirin	21, 22, 29, 30
Dermacentor variabilis	subolesin, GST, ubiquitin, vATPase, selenoproteins M and W2a, hematopoietic stem/progenitor cells protein-like, actin Proteasome 26S subunit, ferritin1, varisin, akirin	15, 19, 22, 24, 26, 30-32
Dermacentor marginatus	subolesin	22
Amblyomma americanum	cDNA library, subolesin, akirin	17, 22, 30
Amblyomma hebraeum	subolesin, voraxin	28
Rhipicephalus sanguineus	Rs86, subolesin	22, 23
Rhipicephalus microplus	GST, ubiquitin, selenoprotein, Bm86, Bm91, subolesin, GI, GIII, EF1a, flagelliform silk protein, von Willebrand factor	16, 18, 25, 27
Rhipicephalus annulatus	ubiquitin, subolesin, EF1a, GIII	16

Table 1. Tick species in which the RNAi protocol has been used.

Figure 1. Placement of ticks, ventral side up, on double sticky tape adhered to a sheet of red dental wax. The ticks are placed in groups of 5, after which a small strip of masking tape is placed over the mouthparts in order to further secure the ticks while allowing the injector to observe the body of the tick during injection.

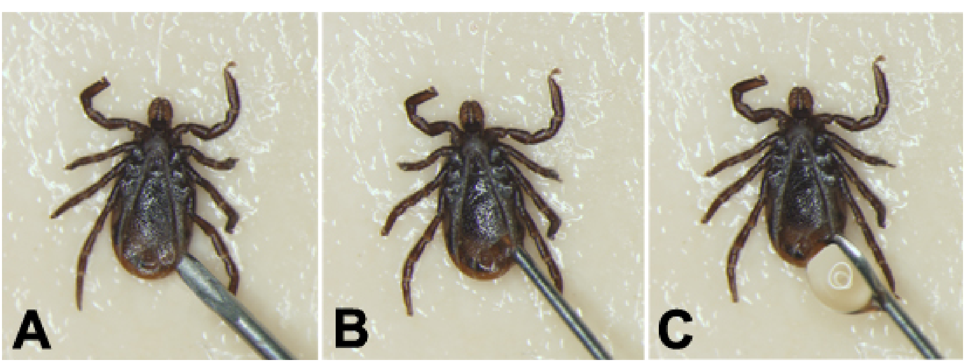

Figure 2. The injection procedure includes (a) piercing the lower right quadrant of the tick exoskeleton with a insulin syringe fitted with a 29 gauge needle in order to create an injection site, (b) immediate injection of the dsRNA at this site using a Hamilton syringe with a 33 gauge needle which (c) most likely will result in some leakage of tick hemolymph/fluids.

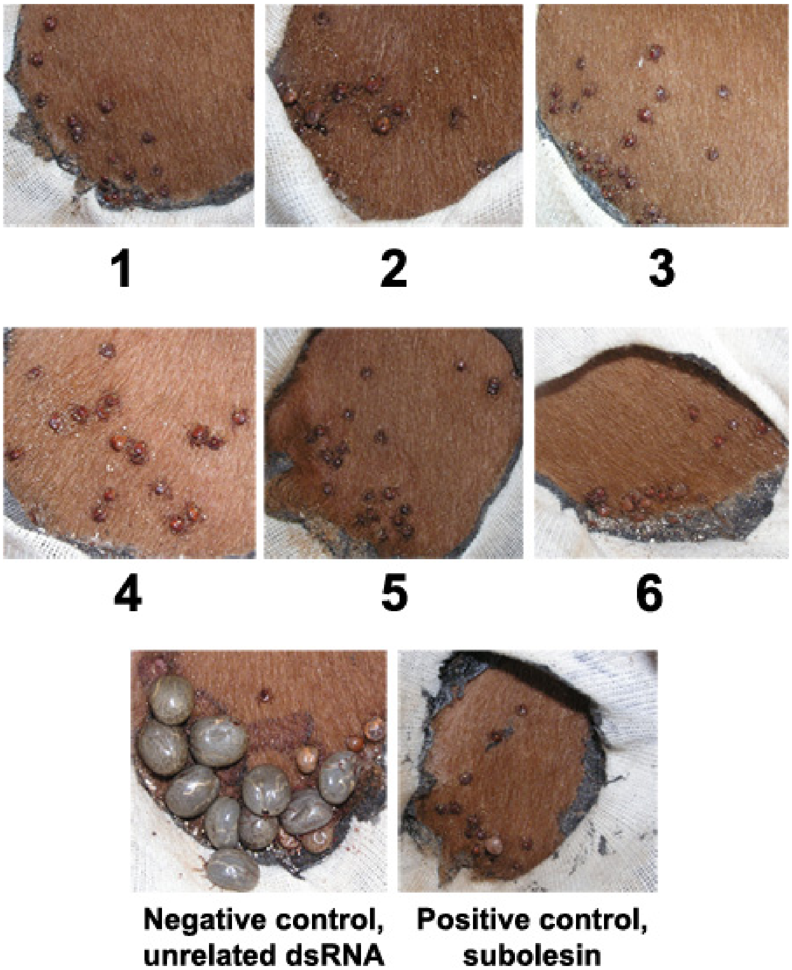

Figure 3. A panel of tick six groups in which RNAi was used to screen for tick protective antigens in Amblyomma americanum. The phenotypic changes in ticks can be seen when compared with the positive subolesin RNAi control and the negative unrelated dsRNA control. In this experiment the effect of RNAi on tick mortality, weights, and oviposition of each group was statistically analyzed.

Discussion

Although other methods have been described for RNAi in ticks[14, 33], the injection of dsRNA described here is the most widely used in both unfed (Table 1) and fed ticks[16,25,34]. RNAi has been shown to be a valuable tool for the study of tick gene function, the characterization of the tick-pathogen interface and the screening and characterization of tick protective antigens[14,35]. In particular, RNAi has become the most valuable tool for functional analyses in ticks[35].

Methodologically, RNAi will likely evolve into more efficient methods that may allow gene knockdown in a large number of individuals. The mechanism of dsRNA-induced RNAi in ticks should be refined to contribute to a better understanding and utilization of this genetic approach in this species[35,36]. The extent of off-target effects of RNAi in ticks is also an important question that needs to be fully addressed[14,27]. Finally, RNAi will most likely provide comprehensive contributions to the study of tick gene regulation and systems biology and the tick-pathogen interface and may have an impact on the development of vaccines to control tick infestations and the transmission of tick-borne pathogens.

Disclosures

No conflicts of interest declared.

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