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GENETICS, EVOLUTION, AND PHYLOGENY - SHORT COMMUNICATION

Genetic diversity of the ATAQ gene in *Rhipicephalus microplus* collected in Mexico and implications as anti-tick vaccine

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Abstract

The cattle tick *Rhipicephalus microplus* has a large impact on cattle production due to its bloodsucking habit and transmission of pathogens that cause babesiosis and anaplasmosis. Application of acaricides constitutes the major control method but is often accompanied by serious drawbacks, including environmental contamination and an increase in acaricide resistance by ticks. The recent development of anti-tick vaccines has provided positive results in the post-genomic era, owing to the rise of reverse vaccinological and bioinformatics approaches to analyze and identify candidate protective antigens for use against ticks. The ATAQ protein is considered a novel antigen for the control of the cattle tick *R. microplus*; it is expressed in midguts and Malpighian tubules of all ticks from the *Rhipicephalus* genus. However, genetic diversity studies are required. Here, the *ATAQ* gene was sequenced of seven *R. microplus* tick isolates from different regions in Mexico to understand the genetic diversity. The results showed that sequence identity among the Mexican isolates ranged between 98 and 100% and 97.8–100% at the nucleotide and protein levels, respectively. Alignments of deduced amino acid sequences from different *R. microplus* ATAQ isolates in Mexico revealed a high degree of conservation. However, the Mexican isolates differed from the *R. microplus* "Mozambique" strain, at 20 amino acid residues. Finally, the analysis of more *R. microplus* isolates, and possibly of other *Rhipicephalus* species, to determine the genetic diversity in the *ATAQ* locus is essential to suggest this antigen as a vaccine candidate that might control tick infestations.

Keywords *Rhipicephalus microplus* · ATAQ · Genetic diversity · Tick vaccine

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Introduction

The cattle tick *Rhipicephalus microplus*, is considered to be the most important ectoparasite in the tropics and subtropics worldwide (Almazán et al. 2010; Popara et al. 2013; Lagunes et al. 2016). Mexico has approximately 34 million head of cattle and it is estimated that about 80% of the cattle production is at risk of ticks and tick-borne diseases (TTBDs). Additionally, economic losses caused by *R. microplus* and TTBDs have been estimated to be US$ 573.61 million per year (Rodríguez-Vivas et al. 2017). Recent efforts to control tick infestations using chemical acaricides have several limitations, including the increased acaricide resistance by ticks, environmental pollution, and contamination of food products with drug residues (Kaewmongkol et al. 2015; Ramírez et al. 2016). Vaccine development is a promising alternative to control tick infestations and pathogen transmission. Currently,

two recombinant Bm86 protein-based vaccines are commercially available in Australia, Cuba, Mexico, and South America (Guerrero et al. 2012; Parizi et al. 2012; Schetters et al. 2016; Blecha et al. 2018). Nevertheless, variable efficacy levels against *R. microplus* strains in separate geographical locations are reported, which may be associated with natural allelic variations in the Bm86 protein and also physiological differences between tick species (Sossai et al. 2005; Blecha et al. 2018). Recently, the ATAQ protein was identified as a putative Bm86 homolog with high similarity; it is expressed in midgut and Malpighian tubules of all ticks from the *Rhipicephalus* genus, which suggests ATAQ as a candidate protein for vaccine development and a potential antigen for the control of the cattle tick *R. microplus* (Nijhof et al. 2010; Aguirre et al. 2016). However, genetic variability has been reported to be one of the reasons for reduced antigenicity in vaccines, making it necessary to obtain information from strains on different geographical regions to develop effective vaccines against infestations by *R. microplus* (Popara et al. 2013; Rodríguez-Mallon 2016). Thus, this study aimed to analyze the *ATAQ* gene in *R. microplus* collected from several geographic locations in Mexico and compare the data with tick species previously reported in order to determine if the sequence variation in the *ATAQ* locus could potentially affect the effectiveness of a possible anti-tick vaccine.

Materials and methods

Ticks

The *R. microplus* (Susceptible "Media Joya," "Huastecas," and "Hybrid") adult female ticks were obtained from laboratory colonies maintained at the CENID-SAI, INIFAP, Mexico. Originally, these tick strains were collected from infested cattle in Tapalpa, Jalisco, and Aldama, Tamaulipas, Mexico. Additionally, semi-engorged *R. microplus* ticks were collected from naturally infested cattle in Candelaria, Campeche; Santiago Ixcuintla (The Ranch: "Verdineño"), Nayarit; Aldama ("Vargas"), Tamaulipas; and Moyahua, Zacatecas, Mexico. Oviposition and hatching by female ticks in humidity chambers at 12-h light: 12-h dark photoperiod, 27 °C and 95% relative humidity occurred at the CENID-SAI, INIFAP. Larvae were used for RNA extraction at 15 days after hatching, resulting in 7 *R. microplus* tick isolates from different geographic locations of Mexico.

RNA extraction and cDNA synthesis

Approximately 100–150 unfed tick larvae from each *R. microplus* tick isolate were used for the experiment. Total RNA was extracted from homogenized tick samples using TRIzol reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Synthesis of cDNA was carried out from 5 μg of total RNA using RevertAid First Strand cDNA Synthesis (Thermo Scientific®). The cDNA was subsequently used as the template to amplify full length *ATAQ* gene (1818 bp) reported for the *R. microplus* "Mozambique" strain (GenBank Accession number GU144589.1).

Amplification and cloning of *ATAQ* gene

Primers were designed to amplify and clone the full-length *ATAQ* gene as follows: forward primer 5′-ATGG GAAGAATGAACAAC-3′ and reverse primer 5′-TCAG GCCTCTTCCTCCGTTG-3′. The PCR was carried out with Platinum Taq DNA polymerase (Invitrogen) at 95 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 60 °C for 1 min and 72 °C for 1 min, and a final extension step at 72 °C for 10 min. PCR products were electrophoresed on 1% agarose gels to check the size of amplified fragments by comparison with a DNA molecular weight marker (1 kb Plus DNA Ladder, Invitrogen). Amplified fragments were purified (Wizard, Promega) and cloned using pCR®4-TOPO TA Cloning® Kit (Invitrogen). The plasmid constructs were used to transform *E. coli* strain TOP 10® (Invitrogen), and the resultant clones were screened with PCR prior to growth in selective broth and plasmid isolation for DNA sequencing. At least five different clones from each PCR product were sequenced to rule out any sequencing errors.

Sequence analysis

Nucleotide sequences were generated in duplicate and both directions using a 3500xL Genetic Analyzer (Applied Biosystems/Hitachi, Forest City, CA). T7 (forward) and T3 (reverse) sequencing primers and the designed internal primers, 5′-ATCCTTACTACAAGTGCAACTGC-3′ and 5′-TGGCACTTTGGATCAGCTTCTC-3′, were used to obtain full-length coverage of the *ATAQ* gene. Nucleotide consensus sequences were obtained from each isolate and then translated using the Molecular Evolutionary Genetic Analysis software package (MEGA 7) (Kumar et al. 2016). Nucleotide and amino acid sequences from each isolate were then aligned using BioEdit Sequence Alignment Editor 7.1.9 to detect polymorphisms among *R. microplus* ATAQ sequences. Amino acid sequences from each isolate were grouped according to identity/similarity using the SIAS tool (http://imed.med.ucm.es/Tools/sias.html).

Phylogenetic analysis

Phylogenetic analysis was based on comparison of the ATAQ sequences obtained and reference sequences of *ATAQ* and *Bm86* genes deposited in the GenBank database. Evolutionary history was inferred using the maximum

	25	35	86	90	177	195	330	352	387	396	424	447	463
R. microplus Media Joya	C	C	G	A	A	G	G	G	G	A	G	C	C
R. microplus Vargas	T	.	A	G	.	.	.	.	.	.	.	.	.
R. microplus Verdineno	T	.	A	G	.	.	.	.	.	.	.	.	.
R. microplus Hybrid	T	.	A	G	.	.	.	.	.	.	.	.	.
R. microplus Campeche	T	.	A	G	.	.	.	C	T	C	.	T	G
R. microplus Huastecas	T	T	A	.	G	A	.	C	T	C	A	T	G
R. microplus Moyahua	T	.	A	.	G	.	A	C	T	C	.	T	G
R. microplus Mozambique	T	.	A	G	.	.	.	.	.	.	.	.	.

	487	531	567	577	706	771	793	795	834	837	881	882	986
R. microplus Media Joya	G	G	A	A	T	A	G	A	A	C	A	C	G
R. microplus Vargas	.	.	.	.	.	.	.	.	.	.	.	.	T
R. microplus Verdineno	.	A	.	.	.	.	.	.	.	.	.	.	.
R. microplus Hybrid	.	.	.	.	.	.	.	.	.	.	.	.	.
R. microplus Campeche	A	.	C	C	.	.	.	.	.	.	G	.	.
R. microplus Huastecas	A	A	C	C	C	G	A	G	C	G	G	T	.
R. microplus Moyahua	A	A	C	C	.	.	.	.	.	.	G	.	.
R. microplus Mozambique	.	.	.	.	.	.	.	.	.	.	.	.	.

	1001	1137	1152	1161	1170	1410	1413	1421	1464	1611	1695	1701
R. microplus Media Joya	A	A	A	A	T	T	C	G	C	C	A	G
R. microplus Vargas	.	.	.	.	.	.	.	.	.	.	C	A
R. microplus Verdineno	.	G	G	.	.	.	.	.	.	A	C	A
R. microplus Hybrid	.	.	.	.	.	.	.	.	.	A	C	A
R. microplus Campeche	.	G	G	T	C	.	.	.	.	A	C	A
R. microplus Huastecas	G	G	G	T	C	G	T	A	G	.	.	.
R. microplus Moyahua	G	G	G	T	C	.	T	.	.	A	C	.
R. microplus Mozambique	.	.	.	.	.	.	.	.	.	A	C	A

Fig. 1 Alignment of *R. microplus* ATAQ nucleotide sequences. DNA sequences are shown in the single letter nucleotide code. Identical nucleotides are shown with dots. Numbers correspond to the *R. microplus* "Media Joya" ATAQ sequence

likelihood method (ML) (Guindon and Gascuel 2003) based on the Kimura 2-parameter substitution model (Kimura 1980). A gamma distribution was used to model evolutionary rate differences among sites with 2 categories and with invariable sites. To test the robustness of branches in phylogenetic trees, 1000 cycles of bootstraps were used (Felsenstein 1985). All evolutionary analyses were conducted in MEGA 7 software.

Results and discussion

In the present study, the complete coding region of the *R. microplus ATAQ* gene was amplified from tick isolates of 7 different geographical locations in Mexico. Nucleotide sequence data reported in this paper were submitted to GenBank (NCBI) with the accession numbers: MF314445.1 (Media Joya), MF314446.1 (Moyahua), MF314447.1 (Huastecas), MG437296.1 (Campeche), MG437297.1 (Hybrid), MG437298.1 (Verdineño), and MG437299.1 (Vargas). Comparison of the ATAQ nucleotide sequences from the Mexican isolates with the "Mozambique" ATAQ sequence showed polymorphisms in 38 positions (Fig. 1), which affected the conservation of a total of 20 residues as shown in the amino acid sequence alignment (Fig. 2). The ATAQ sequences also showed regions of extensive similarity between amino acids 29–110, 193–236, 474–537, and 567–606 among *R. microplus* isolates.

Genetic analysis provides evidence of gene function and genetic variation in ticks, the latter being suggested as a factor in the variable efficacy between tick species and geographical

	9	12	29	110	118	142	155	163	193	236
R. microplus Media Joya	P	A	G	M	E	V	L	D	N	Y
R. microplus Vargas	S	.	E	.	.	.	.	.	.	.
R. microplus Verdineno	S	.	E	.	.	.	.	.	.	.
R. microplus Hybrid	S	.	E	.	.	.	.	.	.	.
R. microplus Campeche	S	.	E	.	Q	.	V	N	H	.
R. microplus Huastecas	S	V	E	.	Q	I	V	N	H	H
R. microplus Moyahua	S	.	E	I	Q	.	V	N	H	.
R. microplus Mozambique	S	.	E	.	.	.	.	.	.	.

	265	278	279	294	329	334	470	474	537	567
R. microplus Media Joya	E	K	D	D	W	Q	N	R	H	M
R. microplus Vargas	.	.	.	.	L	.	.	.	.	I
R. microplus Verdineno	.	.	.	.	.	.	.	.	Q	I
R. microplus Hybrid	.	.	.	.	.	.	.	.	Q	I
R. microplus Campeche	.	.	.	G	.	.	.	.	Q	I
R. microplus Huastecas	K	N	E	G	.	R	K	Q	.	.
R. microplus Moyahua	.	.	.	G	.	R	.	.	Q	.
R. microplus Mozambique	.	.	.	.	.	.	.	.	Q	I

Fig. 2 Alignment of *R. microplus* ATAQ protein sequences. Protein sequences are shown in the single letter amino acid code. Identical amino acids are shown with dots. Numbers correspond to the *R. microplus* "Media Joya" ATAQ sequence

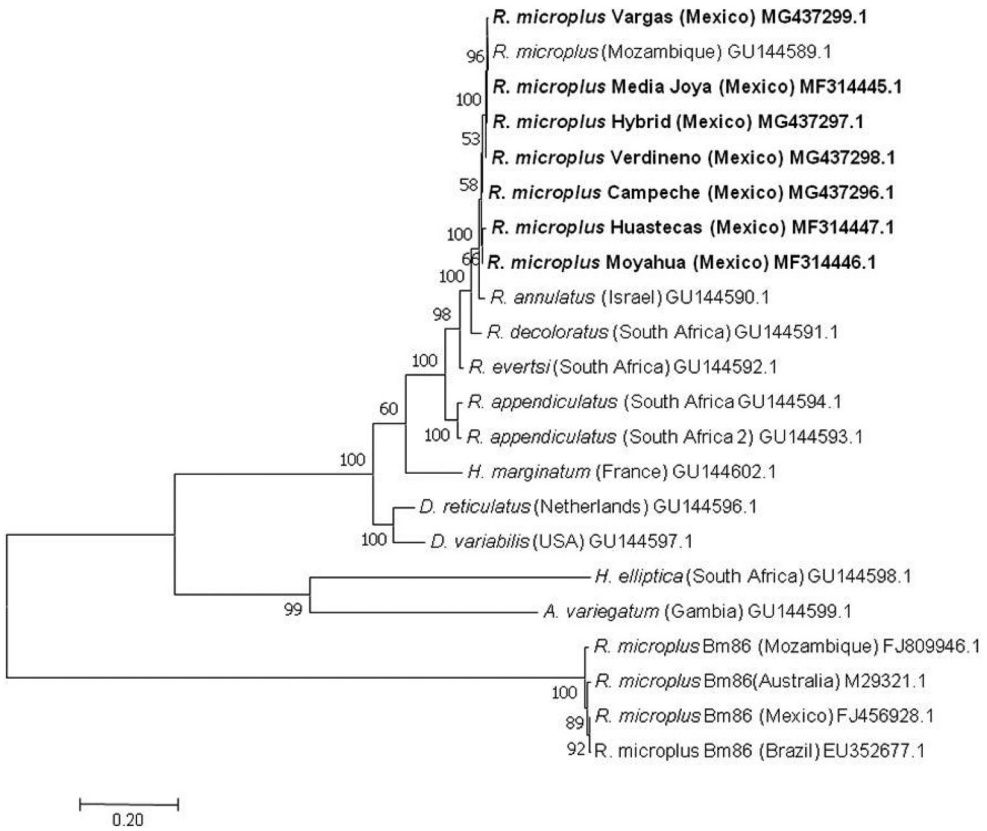

Fig. 3 Phylogenetic relationship of ATAQ sequences. The number represents the percentage of 1000 replications (bootstrap support) at each node of branches. The country of origin and GenBank accession number are indicated. Sequences data generated in the present study are highlighted in bold

Table 1 Identity/similarity Matrix of the ATAQ sequences reported in public database. Nucleotide (below diagonal) and amino acid (above diagonal) identity/similarity for all pairwise comparisons of tick ATAQ sequences

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1. *R. microplus* Media Joya (Mexico)	■	99.3	99.3	99.3	98.6	97.8	98.5	99.3	97	96	89.7	91.2	95.3	77.8	75.9	80.1	42.5	45.5
2. *R. microplus* Vargas (Mexico)	99.6	■	99.6	99.6	99	97.8	98.5	99.6	97	96	89.4	90.9	95	77.8	75.9	80.3	42.3	45.5
3. *R. microplus* Verdineño (Mexico)	99.5	99.7	■	100	99.3	97.8	98.8	100	97.3	96	89.4	91	95	78	76	80.5	42.5	45.5
4. *R. microplus* Hybrid (Mexico)	99.6	99.8	99.8	■	99.3	97.8	99	100	97.3	96	89.4	91	95	78	76	80.5	42.5	45.5
5. *R. microplus* Campeche (Mexico)	98.9	99.1	99.3	99.2	■	98.5	100	99.3	97.6	96.3	89.2	90.9	95.3	78.1	76.2	80.8	42.3	45.5
6. *R. microplus* Huastecas (Mexico)	98.2	98.1	98.2	98	99	■	98.6	97.8	97.3	95.8	89	90.5	95.2	78.1	75.9	80.6	42.2	45.7
7. *R. microplus* Moyahua (Mexico)	98.7	98.7	99	98.8	100	99.1	■	98.8	97.8	96.1	89.4	91	95.5	77.6	75.7	80.6	42.3	45.5
8. *R. microplus* (Mozambique)	99.6	99.8	99.8	100	99.2	98.1	98.8	■	97.3	96	89.4	91	95	78	76	80.5	42.5	45.5
9. *R. annulatus* (Israel)	97.9	97.9	98	98	98.4	98	98.5	98	■	95.3	88.2	89.8	94.3	77.3	75.5	79.86	42.2	45
10. *R. decoloratus* (South Africa)	95.4	95.4	95	95	95.8	95.4	95.7	95.4	95.5	■	90	91.8	95.8	78.5	77	80.6	42.3	45.2
11. *R. appendiculatus* I (South Africa)	89.4	89.3	89	89	90	89.3	89.6	89.3	89.2	90.1	■	96.6	91.5	77.8	76.5	81.8	42	46.3
12. *R. appendiculatus* II (South Africa)	90	89.9	90	90	90	89.9	90.3	90	89.9	90.7	98.5	■	93.5	77	75.5	81.8	44.3	46.1
13. *R. evertsi* (South Africa)	93.8	93.7	93.8	93.7	94.1	93.8	94.2	93.7	93.7	94.7	92.6	93.5	■	78.3	76.4	80.1	42.3	46.1
14. *D. reticulatus* (Netherlands)	77.4	77	77.6	77.6	77.8	77.7	78	77.6	77.6	78.7	79.4	78.1	78.8	■	91.2	78.8	40.7	46.9
15. *D. variabilis* (USA)	75.9	76	76	76	76.2	76.1	76.2	76	76.3	77.2	78.1	77.2	77.4	91.2	■	77	41.2	46.1
16. *H. marginatum* (South Africa)	78.4	78.4	78.6	78.5	78.9	78.6	78.8	78.5	78.5	79.3	80	80	79.5	79.5	78.2	■	42.8	46.1
17. *H. elliptica* (South Africa)	46.5	46.7	46.8	46.8	46.5	46.2	46.4	46.8	46.2	46.5	46.7	47.4	46.8	47.5	46.5	46.2	■	54.2
18. *A. variegatum* (Gambia)	50	49.9	50	49.9	50	49.9	50	49.9	49.7	49.2	49.3	47.3	49.7	49.7	49.9	49	53.5	■

strains in cattle vaccination trials, since ticks from several regions have experienced through different evolutionary processes (García-García et al. 1999). The results were focused on the relationship between *R. microplus* and other tick species. Phylogenetic reconstruction showed that all the Mexican isolates, together with the *R. microplus* "Mozambique" ATAQ, were grouped into one clade; thus, sequences obtained indicate genetic proximity with the "Mozambique" ATAQ sequence (Fig. 3). Moreover, our analysis provides strong evidence of conservation of the *ATAQ* gene among the *Rhipicephalus* genus (100% bootstrap support), similar to results reported in previous studies (Nijhof et al. 2010). The genetic analysis of the *ATAQ* gene indicated that the sequences obtained from the "Media Joya," "Vargas," "Verdineño," and "Hybrid" isolates showed a high degree of identity/similarity (~99.8%) to the "Mozambique" ATAQ sequence. Conversely, "Campeche," "Huastecas," and "Moyahua" isolates showed 98.1–99.2% identity and 97.8–99.3% similarity to the "Mozambique" ATAQ (Table 1). The ML tree inferred from the *ATAQ* gene is consistent with the amino acid tree, displaying identical topology (data not shown). However, sequence identity between *R. microplus* with *Haemaphysalis elliptica* (46.5%) and *Amblyomma variegatum* (50%) was lower than with other species such as *Hyalomma marginatum* (78.4%) and *Dermacentor* spp. (~76%). The phylogeny of ticks obtained with ATAQ and Bm86 sequences was similar to the findings by Nijhof et al. (2010). Additionally, our results agree with previous reports on the evolution of the Bm86 protein family and suggest that the ATAQ proteins have evolved in two gene duplication events through its formation (prostriate and metastriate lineages). The ATAQ protein is an attractive candidate for the development of an anti-tick vaccine due to its anatomical location compared with Bm86. Furthermore, the expression in two vital organs (midgut and Malpighian tubules) of all ticks and its structural similarity to Bm86 suggest that vaccination with ATAQ protein would confer strong protection against multiple tick species.

Recently, Aguirre et al. (2016) designed a synthetic peptide from the *R. microplus* ATAQ protein (GenBank: ADR01301.1), located between residues 531 and 547 (NH2-PQPPHHQKWPFPTTPMA-COOH) with several antigenic properties. The researchers evaluated this peptide against *Rhipicephalus sanguineus* and *R. microplus* with an average protection efficacy of 31%. Interestingly, our results detected a polymorphism within that linear B cell peptide in three Mexican *R. microplus* ATAQ sequences located in the position 537. The peptide contains glutamine (Q) instead of a histidine (H) in "Media Joya," "Vargas," and "Huastecas" isolates. This result might indicate the possible reason why the synthetic peptide elicited a weak protective antibody response in host species and a poor reduction in all parameters evaluated when it was tested in both *Rhipicephalus* ticks. This is consistent with previous experimental trials of tick vaccines (García-García et al. 1999, 2000; Willadsen 2004; Anbrasi et al. 2014). The results clearly suggest that sequence divergence has a direct effect on the antigenicity of vaccines, and it is not enough to use bioinformatics strategies to design a multi-target vaccine directed to the control of tick infestations, without first analyzing the genetic variability of the target and physiological factors that could decrease the efficacy of tick vaccines (Freeman et al. 2010; Popara et al. 2013; Anbarasi et al. 2014). As discussed previously, it is essential to identify and analyze proteins in full detail, and above all, use the genetic material from predominating tick strain in a specific geographical location for the development of anti-tick vaccines (Canales et al. 2009; Moreno-Cid et al. 2013; Shakya et al. 2014).

Conclusion

This is the first report that estimates variation of *R. microplus ATAQ* gene in Mexico, which was demonstrated through genetic analysis among geographic isolates of the same species. Analyses of *ATAQ* gene sequences showed a high degree of conservation among them, suggesting a conservation of antigenic determinants in the ATAQ protein; however, specific polymorphisms were identified, which may be responsible for the variations of antigenic features, and must be taken into account when designing an ATAQ protein-based vaccine. Further research is necessary, exploring genetic and physiological diversity among national tick isolates from other geographical regions and among different tick species. Finally, vaccination experiments with the full recombinant ATAQ protein are essential to suggest this antigen as a vaccine candidate that might control tick infestations.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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