



Detection of specific nucleotide changes in the hypervariable region of 16S rDNA gene of *Rhipicephalus (Boophilus) microplus* and *Hyalomma anatolicum anatolicum*

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ABSTRACT

Hyper variable segment of mitochondrial 16S rDNA from different stages of laboratory reared, disease free and acaricide susceptible *Hyalomma anatolicum anatolicum* and *Rhipicephalus (Boophilus) microplus* were partially amplified, sequenced and analyzed with the aid of the GenBank database. Thirty conserved genus specific nucleotide change were observed in Hyalommid and Boophilid ticks. These conserved sequences were sufficient to identify embryonic stages of the ticks. These conserved sequences at the genus level could act as biomarker for identification of ticks during epidemiological studies of tick borne diseases, transmitted by Hyalommid and Rhipicephalid ticks.

Key words: *Hyalomma anatolicum anatolicum*, *Rhipicephalus (Boophilus) microplus*, 16S rDNA

Hyalomma anatolicum anatolicum and *Rhipicephalus (Boophilus) microplus* are considered to be the most damaging tick species in India, and the control program against tick-borne diseases is specifically focused on these tick species (Ghosh *et al.* 2007). These tick species are being maintained in the entomology laboratory of the institute (Azahianambi and Ghosh 2007) and are under use for various entomological, immunological, biochemical and genetic studies.

Molecular techniques were used for genus level identification of several ticks (Black and Piesman 1994, Crampton 1996, Black *et al.* 1997). The genes were preferred in comparison to ITS1 ITS2 and 18s rDNA segments because the sequences are shorter and more consistent in length, have a higher percentage of alignable bases for sequence comparisons and devoid of any complications caused by the multiple alleles or introns (Mangold 1998). An important aspect of rDNA of ticks is that they have secondary structures

that are moderately well conserved among distantly related taxa (Caetano-Anollés 2002), while hypervariable regions of 450bp is widely variable in different tick species. The hypervariable region is targeted for identification of genus specific conserved nucleotides by amplification and sequencing of this region. Universal primers are usually chosen as complementary to the conserved regions at the beginning of the gene and at either the 540–bp region or at the end of the whole sequence (about the 1,550 bp region), and the sequence of the variable region in between is used for the comparative study.

The aim of current study was to characterize hypervariable segments of the 16S rDNA in hyalommid and rhipicephalid ticks and to identify the specific regions of the hypervariable segment that would be most characteristic for identifying genera specific nucleotide changes amongst the entire tick species study set. The possibility of using the genus specific nucleotide changes as marker to differentiate the tick genera is attempted.

MATERIALS AND METHODS

Tick material: Larvae, partially fed adults, and unfed adults of 3 different generations of laboratory maintained colony of *H. anatolicum anatolicum* and adult of *R. (B.) microplus* were used in the experiment. The different stages of ticks were maintained by rearing on cattle and rabbit hosts as per Ghosh and Azahianambi (2007). The animals were maintained as per the approved guidelines of the Committee

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for Protection, Supervision and Control of Experiments on Animals (CPCSEA), a statutory Indian body monitoring the use of animals in experiments.

DNA extraction and PCR amplification: DNA was extracted from pooled larvae, partially fed adults and unfed adults of laboratory reared *H. anatolicum anatolicum* ticks and adults of *R.(B.) microplus* for amplification and sequencing. Universal primers were used for the amplification of 450bp hypervariable region at 3' end of 16S rDNA gene. The forward primer and reverse primer used were 5'- CCGGTCTGAAGTCAAGTCAAGT-3' and 5'-GCTCAATGATTTTTTAAATTGCTG-3' respectively, originally designed by Norris *et al.* (1996) The PCR conditions was optimized using PTC100 model DNA engine and all the reagents were procured commercially. For 25 µl PCR master mix, the concentration of the reagents were TruStart Hotstart buffer (10X) 2.5 µl, dNTPs (10 mM) 0.5 µl, MgCl₂ (25 mM) 2 µl, forward primer (10 pM) 1 µl, reverse primer (10 pM) 1 µl, TruStart Hotstart Taq polymerase enzyme (5 U/µl) 0.3 µl, template DNA 5.0 µl, nuclease free water 12.7 µl. The PCR amplifications were carried out as follows: an initial denaturation step of 3 min at 94°C, followed by 32 cycles, each of denaturation for 1 min at 94°C, primer annealing for 1 min at 50.5°C and primer extension for 1 min at 72°C. A final extension step was carried out for 15 min at 72°C. Negative controls (no template) were run simultaneously, and reaction mixtures were discarded when any DNA appeared in the control. A 10µl of the reaction mixture was examined in 1.5% agarose gel electrophoresis followed by staining with ethidium bromide, to check the size of amplified fragments by comparison to a DNA molecular weight marker.

DNA sequencing: PCR products were sent to ILS Bioscience and Department of Biochemistry, New Delhi India, for double strand sequencing and confirmation of the sequence information. In direct sequencing of PCR products, the entire gene was sequenced in both the directions.

Sequence analysis: Six *Hyalomma* and 4 *Boophilus* 16S rDNA sequences were aligned with available data base of *R (B) microplus* in the GenBank. using CLUSTAL V method (Higgins *et al.* 1992). No sequence of *H. anatolicum anatolicum* was available in the GenBank database.

RESULTS AND DISCUSSION

PCR amplification of mitochondrial 16S rDNA from acaricide susceptible laboratory reared *H. anatolicum anatolicum* and *Rhipicephalus (B.) microplus* species yielded a fragment of 449 bp and of 454 bp, respectively. The amplified product was directly used for sequencing after purification. To avoid any reading error, both the strands were sequenced. The GenBank accession numbers for these sequences are as follows: Hm176656, Hm176658, Hm176657, GU222462, GU323287, GU323288.

The nucleotide base composition of the sequenced rDNA

hypervariable region showed a high A+T content. It was approximately 77% for all the sequences reported in this study. The average base composition was 39.2% adenine, 9.6% cytosine, 13.8% guanine and 37.4% thymine.

A high sequence similarity of 16S rDNA (99.8% or more) was observed between the different stages of the colony of acaricide susceptible laboratory reared *H. anatolicum anatolicum* tick species. Alignment of sequence data showed that 30 nucleotides across this hypervariable region of mitochondrial 16S rDNA were conserved in *Hyalomma* species while the corresponding nucleotides in *Rhipicephalus* species were different but conserved among *Rhipicephalus* species (Table 1). The nucleotide substitution between *Hyalomma* and *Rhipicephalus* species were found to be transversion. The most frequent transversions were of A→T type (70% of the total substitution).

Ribosomal DNA (rDNA) is the most conserved region of the genome in all the cells. In this study mitochondrial ribosomal 16S DNA sequences were used as an essential tool for identifying and classifying the tick genera. We amplified and reported the first 3 sequences of ribosomal 16S rDNA of *H. anatolicum anatolicum* from different stages of the laboratory reared, acaricide susceptible, homogenous tick colony (Accession nos- Hm176656, Hm176657, Hm176658). In *Hyalomma*, out of 30 genus specific nucleotide, 43% T, 33% A, 13% C, and 10% G, while in *Rhipicephalus*, the corresponding nucleotides were — 33% T, 47% A, 7% C, and 13% G. The high percentage of similarity (99.8% or more) as observed between the different stages of the laboratory reared, *H. anatolicum anatolicum* and *R. (B.) microplus* could be considered as an indicator of genetic homogeneity in the colony of laboratory reared tick species. These tick species is adapted in the laboratory and is continuously rearing for the last 10–12 years. This inbred population maintained genus-specific nucleotide conservation in the hypervariable region along with other species of the genus.

Sequence analysis of conserved housekeeping genes such as the 16S rRNA are increasingly being used for phylogenetic studies (Black and Piesman 1994, Crampton 1996, Black *et al.* 1997, Mangold 1998) but in the present study mitochondrial ribosomal 16S DNA sequences are used as an essential tool for identifying and classifying the tick genera as reported by Labruna *et al.* (2009). The research laboratories where number of tick species are maintained for conducting different types of experiments involving specific vectors and pathogens, a molecular tool for identification of the tick genera at embryonic level will be of great importance. In the epidemiological study, many times embryonic stages of the ticks are collected and the same can be processed and sequenced for comparison with the known sequences before using the sample for isolation of pathogens. Since the tick species cannot be differentiated morphologically at embryonic level, the molecular tool can be a

Table 1. Conservation of nucleotides in *Hyalomma* and *Boophilus* genera

1	90	92	96	99	113	125	141
	T	T	A	A	A	T	<i>C H. a. anatolicum (LHa)</i> Hm176656
	T	T	A	A	A	T	<i>C H. a. anatolicum (UFH)</i> Hm176658
	T	T	A	A	A	T	<i>C H. a. anatolicum (PFH)</i> Hm176657
	T	T	A	A	A	T	<i>C H. dromedari</i> L34306
	T	T	A	A	A	T	<i>C H. lusinaticum</i> Z97881
	T	T	A	A	A	T	<i>C H. marginatum</i> L34307
	A	C	G	T	T	A	<i>T R.(B.) microplus (34th gen)</i> GU222462
	A	C	G	T	T	A	<i>T R.(B.) microplus (35th gen)</i> GU323287
	A	C	G	T	T	A	<i>T R.(B.) microplus (37th gen)</i> GU323288
	A	C	G	T	T	A	<i>T R.(B.) annulatus</i> Z97877
	186	188	190	191	224	227	
	A	G		T	T	C	<i>G H. a. anatolicum (LHa)</i> Hm176656
	A	G	T	T	C	G	<i>H. a. anatolicum (UFH)</i> Hm176658
	A	G	T	T	C	G	<i>H. a. anatolicum (PFH)</i> Hm176657
	A	G		T	T	C	<i>G H. dromedari</i> L34306
	A	G	T	T	C	G	<i>H. lusinaticum</i> Z97881
	A	G	T	T	C	G	<i>G H. marginatum</i> L34307
	T	A	A	A	T	A	<i>R(B.) microplus (34th gen)</i> GU222462
	T	A	A	A	T	A	<i>R.(B.) microplus (35th gen)</i> GU323287
	T	A	A	A	T	A	<i>R.(B.) microplus (37th gen)</i> GU323288
	T	A		A	A	T	<i>A R. (B.) annulatus</i> Z97877
	237	239	252	263	267	271	277 287
	A	C	T	A	T	T	<i>T A H. a. anatolicum (LHa)</i> Hm176656
	A	C	T	A	T	T	<i>T A H. a. anatolicum (UFH)</i> Hm176658
	A	C	T	A	T	T	<i>T A H. a. anatolicum (PFH)</i> Hm176657
	A	C	T	A	T	T	<i>T A H. dromedari</i> L34306
	A	C	T	A	T	T	<i>T A H. lusinaticum</i> Z97881
	A	C	T	A	T	T	<i>T A H. marginatum</i> L34307
	G	T	A	T	G	A	<i>C G R.(B.) microplus (34th gen)</i> GU222462
	G	T	A	T	G	A	<i>C G R.(B.) microplus (35th gen)</i> GU323287
	G	T	A	T	G	A	<i>C G R.(B.) microplus (37th gen)</i> GU323288
	G	T	A	T	G	A	<i>C G R. (B.) annulatus</i> Z97877
290	92	299	323	327	341	353	400
	CTA	T	A	A	T	T	<i>G H. a. anatolicum (LHa)</i> Hm176656
	CTA	T	A	A	T	T	<i>G H. a. anatolicum (UFH)</i> Hm176658
	CTA	T	A	A	T	T	<i>G H. a. anatolicum (PFH)</i> Hm176657
	CTA	T	A	A	T	T	<i>G H. dromedari</i> L34306
	CTA	T	A	A	T	T	<i>G H. lusinaticum</i> Z97881
	CTA	T	A	A	T	T	<i>G H. marginatum</i> L34307
	AAT	A	T	T	A	A	<i>A R.(B.) microplus (34th gen)</i> GU222462
	AAT	A	T	T	A	A	<i>A R.(B.) microplus (35th gen)</i> GU323287
	AAT	A	T	T	A	A	<i>A R.(B.) microplus (37th gen)</i> GU323288
	AAT	A	T	T	A	A	<i>A R.(B.) annulatus</i> Z97877

LHa- larvae, UFH- unfed adult, PFH- partially fed adult of *Hyalomma*.

useful aid for differentiation of species and save the crucial biological material. This method is highly sensitive and since the method is based on mitochondrial DNA amplification, degraded or small amounts of samples can also be analyzed. The universal primer set enables PCR amplification of 16S rDNA sequences in the samples from many tick species for direct sequencing (Norris *et al.* 1996) and the sequences can

be compared with a great stock of registered data in the GenBank. The method can be effectively used in the laboratories working on the epidemiological studies of fatal tick borne human and animal diseases transmitted by *Hyalomma* and *Rhipicephalus* tick species.

We are in the process of analyzing a large pool of sequence data generated in our laboratory which will help in

development of biomarker for identification of tick genera at embryonic stage.

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