

Investigation of Bm86 gene analog sequence in some Iranian populations of *Hyalomma anatolicum* species

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Ticks are an important problem among zoonotic diseases. Main veterinary species of hard ticks are: *Hyalomma* sp. *Boophilus* sp and *Rhipicephalus* sp. Among them *Hyalomma* sp. have widespread distribution in Iran and Middle East. The best method to control *Hyalomma* sp. is immunological one, by using antigens, which causes immunologic and physiologic reactions in host. Bm86 is one of the best antigens, vaccines of which have been produced in Cuba and Australia (TICKGARD, GAVAC). In tracing for Bm86 homologue in *H. anatolicum* and phylogenic research based on the construction and characterization of this gene. *H. anatolicum* populations were collected from various regions of Iran and subjected to DNA extraction. After PCR reaction, comparison sequencing of this gene in *Hyalomma* and *Boophilus* ticks indicated that probably there are some conserved regions inside this gene in DNAMAN program. More differences between sequences of this gene in *H. anatolicum* populations collected from Iran and the one from India show that biogeographical separations affect genomic characters.

Key words: Tick, *Hyalomma anatolicum*, PCR reaction, Bm86 gene, DNA extraction, Phylogenetics

INTRODUCTION

Hyalomma anatolicum anatolicum (Acari: Ixodidae) is the main vector of tropical theileriosis in southern Europe, the middle and Far East including china (De vos and Zeinstra, 2001). Ticks belonging to this genus are well known vectors and avid parasites of man.

One of the most important diseases transmitted by *Hyalomma* ticks is Crimean- Congo hemorrhagic fever (CCHF). *H. a. anatolicum* is widely distributed in parts of the Near East, Asia Minor, southern Europe, southern Russia and India (De vos and Zeinstra, 2001).

Tick and Tick-borne diseases have been studied in many of tropical and subtropical countries of the world. The new method to control cattle tick *Boophilus microplus* has been developed in Australia and South America. This method has focused on a concealed membrane glycoprotein of *Boophilus* gut lumen cells called Bm86 (De la Fuente and Garsia, 2000, Garsia and Montero, 2000). Antibodies produced in vaccinated cattle recognize Bm86 in the gut of the tick and cause damages by a mechanism that is not yet well understood (De vos and Zeinstra, 2001). Although, it has been associated with the process of Endocytosis (Riding et al, 1994).

As the major tick in Iran seems to be *H. a. anatolicum* in tracing for Bm86 analog in *H. a. anatolicum* (De vos and Zeinstra, 2001) based on the construction and characterization of this gene,

we collected *H. a. anatolicum* populations from various regions of Iran. Studying different populations of Iranian *H.a.anatolicum* and comparison with their gene structure can explain weather biogeographical separation and different climate conditions have affected the genomic structure of the tick or not.

Our objective is studying sequence of Bm86 gene analogue in Iranian *H.a.anatolicum* ticks and comparison with the gene fragments among collected populations of this species.

MATERIAL AND METHODS

TICK STRAINS

Adult *H.a.anatolicum* isolated from domestic animals in Booshehr province in south of Iran, Kordan in west of Tehran, and Boein Zahra in south of Tehran. The samples were collected in eppendorf tubes. Fertilized females were laying eggs in tubes and eggs were incubated in 4°C before use.

DNA EXTRACTION

Total DNA was extracted from 0.5 gram eggs *Hyalomma anatolicum anatolicum* or one male tick and purified. DNA was isolated by two methods: chloroform- isoamylalcohol method and Roche company kit.

In chloroform- isoamylalcohol method, before extraction, eggs or a male tick were frozen in liquid nitrogen and homogenized in an extraction buffer (Tris 10 mM, EDTA 1mM, NaCl 100 mM, pH 8.0, SDS 1%). After gentle mixing, proteinaseK was added at a final concentration of 200µg/ml. Samples were incubated for two hours at 37°C , extracted twice with phenol and once with chloroform, precipitated with ethanol and resuspended in 50µl water.

For PCR reaction with genom fragments; 5 primer sets were designed as follow:

Primer name	Primer sequence
155U19 515L19	5'- AGA GGA TGA TTT CGT GTG C-3' 5'- AAG CAG GTT TTT CTC GCA G- 3'
515U18 1046L20	5'- CTG CGA GAA AAA CCT GCT-3' 5'- CCG TGT AGT AAT ACT CAT TC-3'
1046U20 1324L20	5'- GAA TGA GTA TTA CTA CAC GG-3' 5'- CTC TTC TTC GAT TCC TGT TG-3'
1324U20 1915L18	5'- CAA CAG GAA TCG AAG AAG AG-3' 5'- CTG ACT TTC CCG GAT CTG-3'
FBm86C 5RBm86C	5'-TGG AGA TGT GCA GCC CAC CT-3' 5'- ATC CAG ATC CCG GAA AGT C-3'

PCR PROGRAM

Master cycler gradient (eppendorf) was programmed as follow:

5 min at 95°C, 1min at 49°C, then 30 cycle of 1.5min at 72°C , ending with 10 min at 72°C.

Amplified fragments sent to MWG Company for sequencing by Sanger method.

RESULTS

Primer designing is the base of gene separation. As there are no records of this gene sequence in NCBI, we had to design our primers. Search in GeneBank and molecular informatics sites showed that there are only two international report of full length sequence of Bm86 and Bm95 genes from Argentinean *Boophilus microplus*, and there is only one report of Indian *Hyalomma. anatolicum anatolicum* Bm86 gene sequence.

Because of some morphological similarity between Indian and Iranian populations of *Hyalomma anatolicum* species we designed the primers on the basis of Indian population genome. By using DNAMAN and Oligo programs, 5' site were selected along the 2.2Kbp genome that considered as fine candidate to primer designing. DNA template Primers could amplify 600bp, 1Kbp, 500bp, and 300 bp bounds from 5' to 3' side of genome.

We amplify all genes from DNA template. According to the results of first and second set of primers have repeatable bound formation among all primer sets (fig.1and fig.2). These two set can create good bounds from DNA that has no relation to biogeographical site of sampling. Thus, constancy of this part of genome is very high (fig.2).

Third set has not constant output in DNA template and usually there were no result. Therefore, in this part of Iranian population genomic DNA differences are insignificant comoart, probably, with Indian population genomic DNA.

Forth set has hard set up condition (fig.3). This primer interestingly could amplify only with Booshehr samples. In spit of our attempt to set up it for Kordan and Boein Zahra samples, no results were found.

Fifth set could amplify a 300bp bound that has no additional bound and dimmer primer was very low (fig.4). Repetition of this bound has no constancy and more studies showed that there is no relation in bound formation and geographical area of sampling.

After determination of 1600bp sequence of 5' side of gene, the sequence were analyzed by a complete panel of restriction enzymes in DNAMAN program to obtain sites those could be distinguish by means of enzymes. Different mutation points among genome of Booshehr, Kordan and Boein Zahra ticks can be as a result of Biogeographical and climate changes.

DISCUSSION

Exact identification of related species and subspecies is an important problem for taxonomists. In Acarology there are no safe ways about this matter. Numerous papers showed taxonomical studies on mites (Fenton et al, 1995, Gutierrez, 1987, Hart et al 1990, Palmer and Norton 1992). Using of molecular methods in systematic has been introduced by Hillis et al (Hillis et al, 1996).

DNA sequencing technology has efficient role in tick phylogenic analysis (Cruickshank, 2002). Our final goal was studying about differences among individuals belong to one species in focus on Bm86 gene analog sequence, and investigation of probability of biogeographical separation, thus every sample was examined separately. Tick eggs and adult ticks that have not feed on host were selected, because blood components can act as a preventing of performing correct PCR cycles.

For more taxonomic studies and exact identification, we have to do PCR reaction by ticks that were separated of host (Black and Piesman, 1994).In many studies that were done about molecular systematic, some genome specific regions such as ITs and CoI were used and in some cases, genomic differences among species of one genus is very low (Navajas et al, 1996, Navajas et al, 1997).

For studying *Hyalomma anatolicum* populations, Bm86 gene has considered as a indicator and were sequenced analogs, investigated point mutations and then the amount of similarity and differentiation between among individuals of *Hyalomma anatolicum* tick species were estimated.

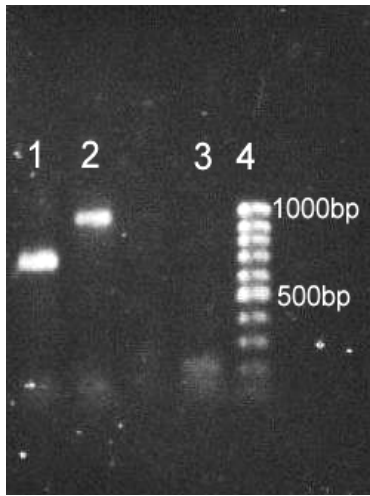


FIG. 1.-

1) First bound 600bp by the first primer 155U, 515L; 2) second bound 1000bp with primer 515U, 1046L; 3) negative control; 4) 1kb Ladder

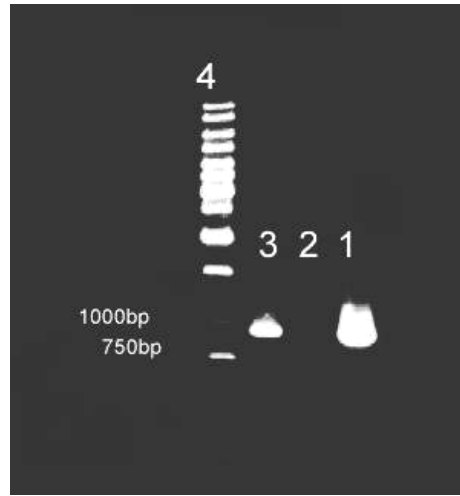


FIG. 2.-

1) 1000bp bound from Booshehr tick DNA; 2). Negative control; 3) 1000bp bound from Boein Zahra tick DNA; 4) 1Kbp Ladder

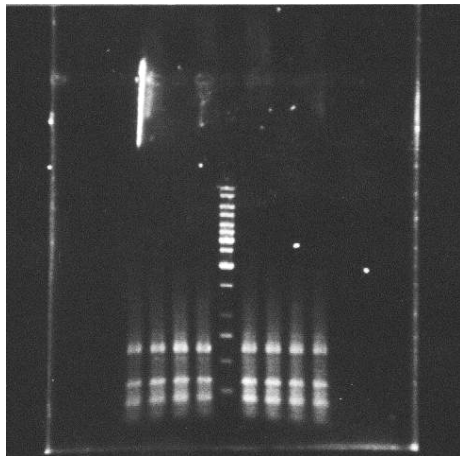


FIG.3.-

Temperature gradient for deletion of dimmer primer and additional bounds In 500 bp bound of primer 1324U, 1915L

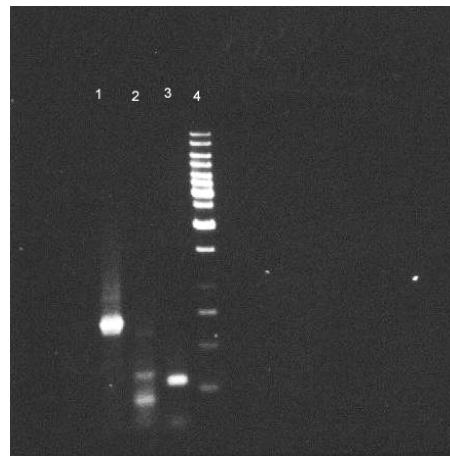


FIG.4.-

1) 500bp bound by 155U, 515L primer; 2) 300bp bound by 1324U, 1915L primer; 3) 300bp bound by FBm86C, RBm86C primer 1Kbp Ladder

De la Fuente (2000) studied this gene in *Boophilus microplus* and reported that there is an intron site in 1700 bp weight with no phylogenical importance (De la Fuente and Garsia, 2000). While, our results showed that in *Hyalomma anatolicum* there are probably two intron site. Also De la Fuente found that *Boophilus microplus* intron site is in 3' side of gene, whereas our results showed Introns in 5' side of *Hyalomma anatolicum* Bm86 gene.

The only report that is available about sequencing of Bm86 analogue in *H.anatolicum* is about Indian samples (De vos and Zeinstra, 2001) that are accessible in NCBI. Because of differences between climate condition of Iran and India comparison sequencing can answer an important question: whether variety in climate could be effective on tick genome structure or not? Using DNAMAN program we found this similarity is very low.

The more sensitive part of work was comparison of gene fragments among samples those were collected from various region of Iran that can show whether biogeographical subspecies are available in this species or not?

As one can see, similarity in primary nucleotides between primers 155U-1046L in Booshehr and Boein Zahra samples is very high that show accessible conformity. The third set of primers has no constant output with DNA template probable in this site variation in nucleotide arrangement which was very high and with forth set of primers only Booshehrs samples were able to bound creation.

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