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Molecular and Phylogenetic Study of Bm86 Gene Ortholog from Hyalomma excavatum Tick from Tunisia: Taxonomic and Immunologic Interest

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Abstract

In order to assess the taxonomic and immunologic interest of Bm86 orthologs of *Hyalomma* ticks, partial sequence of Bm86 gene was amplified and sequenced. The sequences were isolated from three engorged *Hyalomma excavatum* females (Ariana strain, Tunisia). The analysis of nucleotide sequences showed increasing diversity rates of 0.26, 2.36, 4.97 and 6.02% between analyzed sequences and those isolated from *H. excavatum* specimen from a laboratory colony (Sousse strain, Tunisia), *H. anatolicum*, *H. marginatum* and *H. scupense*, respectively. The phylogenetic study showed a perfect agreement with recent data of systematic of ticks. This proves that genetic analysis of Bm86 orthologs isolated from *Hyalomma* ticks could be used to assist morphological diagnosis. In addition, amino-acid sequence comparison showed a high diversity rate (33-34%) between Bm86 and He86-A1/A2/A3 (Ariana strains) which can decrease the effectiveness of vaccination by commercial and experimental vaccines based on Bm86 against *H. excavatum*. Amino-acid diversity between Hd86-A1 used in an experimental vaccine against *H. scupense* and He86-A1/A2/A3 (Ariana isolates) was more limited (10.2%), thus suggesting that Hd86-A1 vaccine candidate might be more appropriate to target H. excavatum tick than corresponding Bm86 vaccines.

Keywords: *H. scupense*; *Hyalomma*; *Rhipicephalus microplus;* DNA sequencing

Introduction

In Tunisia, Ixodids from *Hyalomma* genus represent the most widely distributed tick species [1-3]. Beside their direct pathogenic effects, these species are vectors of important diseases of livestock and in some instances of zoonoses [3,4]. In fact, two species, namely *Hyalomma scupense (H. detritum*) and *Hyalomma dromedarii*, are considered as vectors of tropical theileriosis, caused by Theileria annulata protozoa in the Maghreb [4,5]. Furthermore, *Hyalomma excavatum* is a one of the natural vectors of *Babesia ovis* [6,7], it can also transmit Anaplasma marginale and Anaplasma centrale to cattle under laboratory conditions [8]. Moreover, Hyalomma marginatum transmits the protozoan Babesia caballi causing equine babesiosis [9] and Crimean-Congo hemorrhagic fever virus [10,11].

The morphological identification of Ixodid ticks, particularly *Hyalomma* species, is sometimes difficult [12]. Separate identification keys are used for larvae, nymphs and adult ticks [13,14]. In fact, using morphological characters for species identification could be sometimes imprecise due to several factors like intraspecific variations, the damage of capitulum and adjacent structures during attached tick removal and morphological changes of the surface body of engorged ticks [12,15].

The availability of alternative tools including molecular markers might be useful in this context. It was shown that these markers such as the mitochondrial (mt) 16S ribosomal RNA (rRNA) gene and the internal transcribed spacer 2 (ITS2) are reliable indicators of the phylogeny even at the intraspecific level [16-21]. However, only few molecular studies were performed on *Hyalomma* genus [15,22].

In 1989, the Bm86 gene was isolated from *Rhipicephalus microplus* [23]. A cDNA contained a 1982 pb open reading frame revealed that Bm86 consists of 650 amino acids, including a 19-amino acid signal sequence and a 23-aminoacid hydrophobic region adjacent to the carbonyl terminus. The Bm86 glycoprotein is located on the luminal surface of the plasma membrane of tick gut epithelial cells it is able to stimulate a protective immune response in cattle against subsequent tick infestation [23]. The phylogeny of this gene has been mainly studied in *Rhipicephalus* genus and obtained concordant results with data of the ticks' systematic [24].

Anti-tick cattle vaccination using the Bm86 protein provides an alternative solution to acaricides avoiding human, animal and environment risks [25]. Bm86 vaccines confer a partial cross-protection against *H. anatolicum* and *H. dromedarii*, but were not effective against *H. scupense* and *H. excavatum* [26,27]. The recombinant Hd86-A1, the Bm86 ortholog in *H. scupense*, was cloned, expressed and successfully experimented as a candidate vaccine [27,28]. It has been shown that the Hd86-A1 antigen induced a higher protection against H. scupense than did Bm86 [27].

Based on a calculation of mutation fixation index applied to a partial sequence of 35AA of Bm86 protein, [29] showed that the efficacy of the immunisation with Bm86 protein is inversely proportional with variations in the amino-acid sequence of the antigen in target ticks. It was suggested that an amino acid sequence divergence greater than 2.8% would result in a vaccine efficiency decrease [29]. Thus, in regions where Bm86 based vaccines are expected to cover different tick species, investigations on the extent of diversity occurring between the candidate vaccine sequence and its orthologs in distinct tick species represent an important step toward a rational vaccine development strategy.

The objectives of this study were to explore the possibility of using a partial sequence of Bm86 orthologs for taxonomic purposes of engorged *Hyalomma* spp. female ticks. In addition, we compared

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revealed He86-A sequences from North Tunisian *H. excavatum* population to Bm86 and Hd86-A1 orthologs used in commercial and experimental vaccines.

Materials and Methods

Sampling, obtaining tick tissues and total RNA extraction

Three engorged female *H. excavatum* ticks were collected from three traditional farms located in El Hessiene region (Locality of Raoued, Governorate of Ariana, North Tunisia) characterized by high *Hyalomma* spp. cattle burdens, especially *H. scupense* [3,30]. *H. excavatum* ticks identified with the key of Walker *et al.* (2013) were dissected; fifteen mg of guts were collected and conserved in 800 µl of Trizol (Invitrogen) at -80°C until used. Total RNA was extracted with Trizol reagent (Invitrogen) according to the given instructions.

Reverse transcription and amplification of the target sequences

The first strand synthesis reaction was carried out employing the SuperScript First-strand Synthesis System for RT-PCR kit (Invitrogen, USA), following the manufacturer's instructions. Nucleotides 92–538 from *He*86 gene in *H. excavatum* (according to the Bm86 coding region reference sequence; GenBank Accession number M29321) [23] was amplified by PCR using Hd86F [5'-TCATCCATTTGCTCCGACTTCGG-3'] and Hd86R [5'-AAGCAGGTTTTTCTCGCAGAG-3'] primers designed from Hd86-A1 nucleotide sequence (GenBank Accession number HQ872020) [28]. These primers are conserved within all Bm86 *Hyalomma* sequence orthologs found in Genbank. PCR reactions without cDNA were performed to identify possible contaminations. For each sample, two independent amplification reactions were performed as follows: 1x PCR buffer 5x, 2 mM $MgCl_2$, 0.2 mM dNTPs (Promega, USA), 2 µl cDNA, 0.5 µM of the primers, 0.125 U/µl GoTaq Flexi DNA Polymerase (Promega, USA) and sterile milliQ water in sufficient quantity for 50 µl. Thermal cycling reactions was performed in an automated DNA thermal cycler (GeneAmp PCR System 2700, Applied Biosystems, USA) using the following conditions: an initial step of 2 min at 94°C followed by 40 cycles of a denaturing step of 1 min at 94°C, an annealing step of 1 min at 55°C, an extension step of 1 min at 72°C and a final extension step of 72°C for 7 min. PCR products were electrophoresed in 1% agarose gels to check the size of amplified fragments by comparison to a DNA molecular weight marker (1 kb Plus DNA Ladder, Promega, USA).

DNA sequencing and data analysis

The PCR products were purified after electrophoresis in 1% agarose with the Wizard™ SV Gel and PCR Clean-up System kit (Promega, USA) and directly sequenced in both directions, using Hd86F and Hd86R primers, a conventional Big Dye Terminator cycle sequencing ready reaction kit (Perkin Elmer, Applied Biosystems, Foster City, CA) and an ABI373 automated DNA sequences. Three sequences corresponding to He86-A1 (Ariana isolate 1), He86-A2 (Ariana isolate 2) and He86-A3 (Ariana isolate 3) were submitted to GenBank, they can be retrieved under accession numbers HQ992990, HQ992991 and HQ992992, respectively.

Nucleotides and amino-acid deduced sequences of the Bm86 ortholog from *Hyalomma* sp. were compared with the existing sequences isolated from *Hyalomma* and *Rhipicephalus* spp. ticks using the DNAMAN program (Version 5.2.2; Lynnon Biosoft, Quebec, Canada). The second epidermal growth factor (EGF)-like full domain of Bm86 ortholog sequences of studied *H. excavatum* ticks were identified manually according to the following EGF like pattern "Cys-Xaa3-9-Cys-Xaa3-6-Cys-Xaa8-11-Cys-Xaa0-1-Cys-Xaa5-15-Cys" [24] (Figure 1). Phylogenetic relationships were determined by nucleotide and amino acid neighbor-joining trees generated using the method of Saitou and Nei [31,32] (Figure 2).

Results and Discussion

Hyalomma ticks are important pests of livestock with major medical and veterinary significance in North Africa [1]. In order to assess the taxonomic and immunologic interest of Bm86 orthologs of *Hyalomma* species, partial sequence of 382 base pairs of Bm86 gene isolated from *H. excavatum* (Ariana strain, Tunisia) was amplified and sequenced. This cDNA fragment that codes for 127 amino acids corresponds to 20.8% of the total nucleotide sequence (Figure 1). The analysis of

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Bm86 (Aus)  : SDFGNEFCRNAECEVVPGAEDDFVCKCPRDNMYFNAAEKQCEYKDTCKTRECSYGRCVESNPSKASCVCE :   70
Bm95 (arg)  : SDFGNEFCRNAECEVVPGAEDDFVCKCPRDNMYFNAAEKQCEYKDTCKTRECSYGRCVESNPSKgSCVCE :   70
Bm86 (moz)  : SDFGNEFCRNAECEVVPGAEDDFVCKCPRDNMYFNAAEKQCEYKDTCKTRECSYGRCVESNPSKASCVCE :   70
Hd86-A1 (Tun) : SDFGkqFCqsAECEViPGtEDDFVCKCPkDdMYyNAAEKkCEYKrTCKTvECSYGhCiEvgPgrtgCgCq :   70
He86-A1 (Tun) : SDFGkqFCqNAECEViPGtEDDFVCKCPkDdMYyNAAEKQCEYKrTCKTvECSYGnCVqisPgrtdCgCq :   70
He86-A2 (Tun) : SDFGkqFCqNAECEViPGtEDDFVCKCPkDdMYyNAAEKQCEYKrTCKTvECSYGnCVqisPgrtdCgCq :   70
He86-A3 (Tun) : SDFvkqFCqNAECEViPGtEDDFVCKCPkDdMYyNAAEKQCEYKrTCKTvECSYGnCVqisPgrtdCgCq :   70
Consensus     sdf   fc  aecev pg ed fvckcp d my naa k ceyk tckt ecsyg c    p    c c

Bm86 (Aus)  : ASDDLTLQCKIKNDYATDCRNRGGTAKLRTDGFIGATCDCGEWGAMNMTTR.NCVPT :   126
Bm95 (arg)  : rSDDLTLQCKIKNDYATDCRNRGGTAKLRTDGFIGATCDCGEWGAMNkTTR.NCVPT :   126
Bm86 (moz)  : ASDDLTLQCKIKNeYATDCqNRGGTAKLRTDGFIGATCDCGEWGAMNkTTR.NCVPT :   126
Hd86-A1 (Tun) : gvDtLTLkCgIqewfAnDCgrRGGTAvLRTDGFlGArCDCGEWGkMNkaqdgkCVPT :   127
He86-A1 (Tun) : gvDtLTLkCgIqewYAnDCgrRGGTAvrRTDGFlGprCDCGEWGkMskgqdgkCVPT :   127
He86-A2 (Tun) : gvDtLTLkCgIqewYAnDCgrRGGTAvrRTDGFlGAryDCGEWGkMskgqdgkCVPT :   127
He86-A3 (Tun) : gvDtLTLkCgIqewYAnDCgrRGGTAvrRTDGFlGArCDCGEWGkMskgqdgkCVPT :   127
Consensus     d ltl c i    a dc  rggta  rtdgf g   dcgewg m       cvpt
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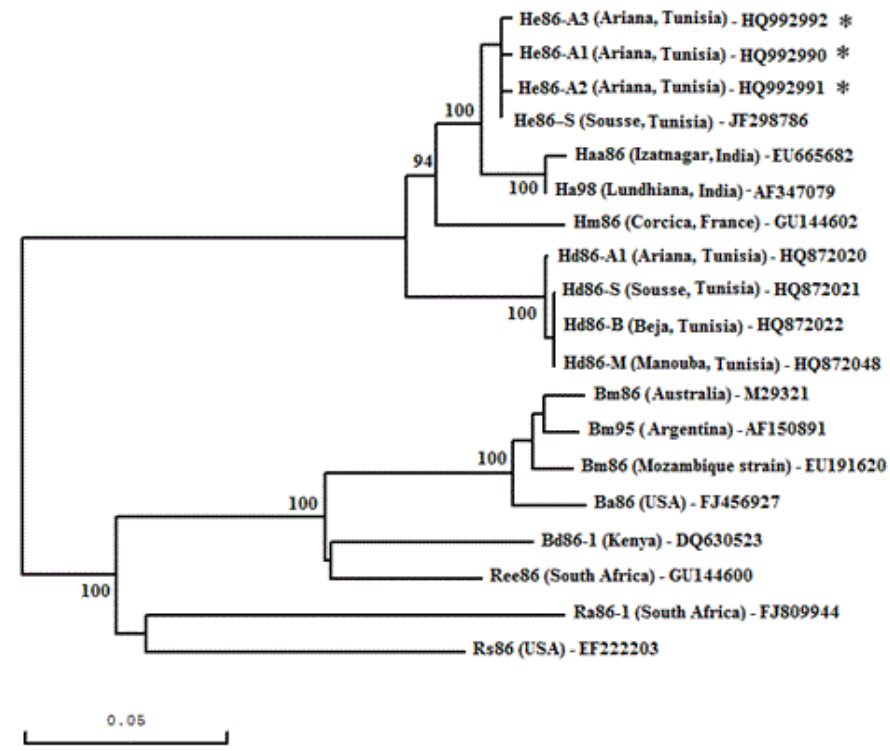
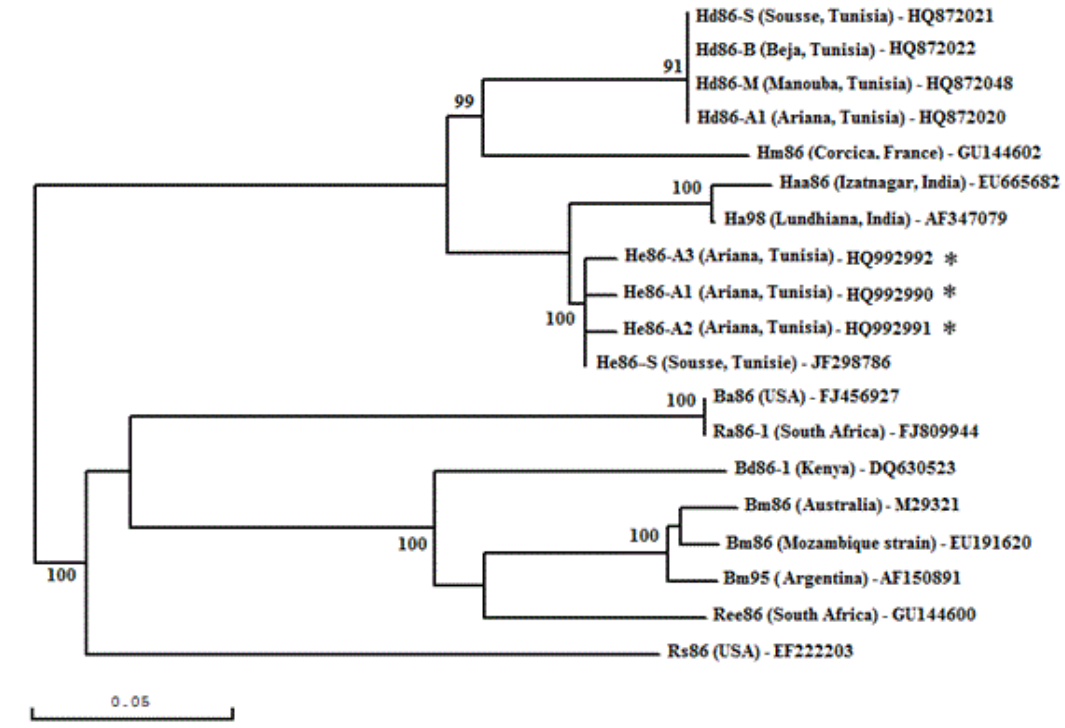
Figure 1: Multiple alignment of amino acid sequences of studied fragment of Bm86 homologue proteins in analyzed specimens of *Hyalomma* sp. with Bm86 amino acid sequences used in commercial vaccines such as Bm86 (Aus) (Australian strain of *Rhipicephalus microplus*, GenBank accession number M29321) and Bm95 (Arg) (Argentine strain of *R. microplus*, GenBank accession number AF150891) and experimental vaccines such as Bm86 (Moz) (Mozambique strain of *R. microplus*, Genbank accession number EU191620) and Hd86-A1 (Tunisian strain of *H. scupense*, Genbank accession number HQ872020).

Second full EGF-like region fitting the pattern "Cys-Xaa3-9-Cys-Xaa3-6-Cys-Xaa8-11-Cys-Xaa0-1-Cys-Xaa5-15-Cys" identified by Nijhof *et al.* (2010) with Xaa is any amino acid except cysteine shaded in gray.



B



Note: Hd: *H. scupense*; He: *H. excavatum*; Hm: *H. marginatum*; Ha: *H. anatolicum*; Bm: *R. microplus*; Ba: *R. annulatus*; Bd: *R. decoloratus*; Ra: *R. appendiculatus*; Ree: *R. evertsi evertsi*; Rs: *R. sanguineus.*

Figure 2: Phylogenetic trees using Neighbor-joining method based on nucleotide sequences of studied fragment of *Bm*86 gene (A) and corresponding proteins (B) in three specimens of *Hyalomma* ticks (marked with an asterisk) and in other of *Rhipicephalus* ticks generated with DNAMAN program (Version 5.2.2; Lynnon Biosoft, Quebec, Canada). Numbers associated with nodes represent the percentage of 1000 bootstrap iterations supporting the nodes (only percentages greater than 50% are represented). The country of origin from each strain and GenBank accession number are indicated.

the second full EGF like region found in Hsp86-A1/A2/A3 shows a great conservation with that of its Bm86 counterpart and confirms its status as Bm86 homolog in the studied *Hyalomma* sp. (Figure 1). In agreement to Ben Said et al., the conservation of this domain reveals the importance of this domain type in the structure and function of this protein.

Sequences of all studied specimens were compared. Nucleotide and amino-acid diversity rates were 0.52 and 1.57% between studied specimens, respectively (Table 1). Indeed, three nucleotides variations at positions 23, 328 and 335 were identified giving three amino acid variations at positions 8, 110 and 112 (Table 2). For comparative sequence analysis, we selected available sequences of *H. excavatum* (Sousse strain) [33], *H. anatolicum* [26,34-36], *H. scupense* [28], *H. marginatum* [34] and *R. microplus* [23,34,37]. The analysis of nucleotide sequences showed increasing diversity rates of 0.26; 2.36; 4.97 and 6.02%, between analyzed sequences and those isolated from *H. excavatum* (Sousse strain, Tunisia), *H. anatolicum*, *H. marginatum* and *H. scupense* respectively (Table 1). The nucleotide diversity of Hsp86-A1/A2/A3 was estimated to 27% when compared to the Bm86 sequences of *R. microplus* (Australia, Argentina and Mozambique) [23,35,37] (Table 1).

Based on nucleotide sequences of studied fragment of Bm86 gene in three specimens of *H. excavatum* Ariana strain and in other species belonging to *Hyalomma* and *Rhipicephalus* genera, a phylogenetic tree was constructed (Figure 2A). *Hyalomma* and *Rhipicephalus* ticks can be classified each one into a separate cluster. The *Hyalomma* cluster is more homogeneous than those of Rhipicephalus ticks. The phylogenetic tree demonstrated that the He86-S isolated from *H. excavatum* (Sousse strain) is more closely related to He86-A1/A2/A3 sequences isolated from Ariana strain of *H. excavatum* by comparison to all other sequences from *Hyalomma* species (Figure 2A). This phylogenetic analysis results were in perfect agreement with insights in the systematic of *Hyalomma* genus ticks [38]. All these data confirm that studied fragment of the Bm86 gene could be used as a molecular aid to *Hyalomma* ticks identification.

Commercial and experimental concealed antigen Bm86 anti-tick based vaccines developed in Australia, Mozambique, Cuba and Tunisia have variable efficacy against *H. anatolicum*, *H. dromedarii*, *H. scupense* and *H. excavatum* [26,27]. This variation could be explained by the variability in protein sequence between the recombinant Bm86 vaccine and Bm86 orthologs expressed in different *Hyalomma* species [27,34]. Accordingly, we have compared revealed sequences from Ariana strain of *H. excavatum* to Bm86 and Hd86-A1 orthologs used in commercial and experimental vaccines. Diversity rates of amino acid sequences of He86-A1/A2/A3 with the Bm86 proteins from *R. microplus* (Australia, Argentina and Mozambique) ranged between 33 and 34% (Table 2). In addition, the obtained amino-acid sequences of He86-A1/A2/A3 were compared with the Hd86-A1 sequence from *H. scupense* candidate vaccine [28]. The amino acid diversity was estimated to 10% and showed 25 different nucleotides (Table 3). This variability must be accounted as one of the major factors conditioning the efficacy of Bm86 commercial vaccines when used against different *H. excavatum* strains. Although, epitopes inducing protective immune responses in cattle are not extensively characterized in Bm86 protein, the extent of diversity in He83-A1/A2/A3 compared to Bm86 proteins confirmed by phylogenetic study (Figure 2B) is highly relevant in term of vaccine development strategy since the Hd86-A1 protein might be expected to be more effective than Bm86 commercial vaccines against different Tunisian *H. excavatum* populations.

However, [27] showed that cattle vaccination with Hd86 did not protect against *H. excavatum* adult infestations. A low expression of He86 glycoprotein in adult *H. excavatum* ticks could explain this result. Indeed, Hd86 expression levels significantly decrease, following moulting of *H. scupense* nymphs [37], whereas continuous expression of Bm86 was reported during the life cycle of *R. microplus* [35]. Consequently, a vaccination trial using a recombinant Hd86-based vaccine of cattle against immature *H. excavatum* ticks and a quantification of He86 mRNA expression levels in different *H. excavatum* stages are needed in order to validate the use of Hd86-based vaccine in integrated tick control strategies in cattle.

Authors' Contributions

MBS designed and performed molecular biology experiments, analyzed the data, and wrote the manuscript. MM participated in molecular biology experiments. MG and YG participated in design study and draft the manuscript. LS and MJ assisted to collect ticks and obtain tissues. MAD conceived the study, and participated in its design

Bm86 sequences	He86-A1 (Tun)		He86-A2 (Tun)		He86-A3 (Tun)	
	Nucleotide mutations /total (%)	Amino acid mutations /total (%)	Nucleotide mutations /total (%)	Amino acid mutations /total (%)	Nucleotide mutations /total (%)	Amino acid mutations /total (%)
He86-A2 (Tun)	2/382 (0.52)	2/127 (1.57)	-	-	2/382 (0.52)	2/127 (1.57)
He86-A3 (Tun)	2/382 (0.52)	2/127 (1.57)	2/382 (0.52)	2/127 (1.57)	-	-
He86-S (Tun)	1/382 (0.26)	1/127 (0.78)	1/382 (0.26)	1/127 (0.78)	1/382 (0.26)	1/127 (0.78)
Ha98 (Lud)	9/382 (2.36)	6/127 (4.72)	9/382 (2.36)	6/127 (4.72)	9/382 (2.36)	6/127 (4.72)
Haa86 (Iza)	11/382 (2.88)	8/127 (6.30)	11/382 (2.88)	8/127 (6.30)	11/382 (2.88)	8/127 (6.30)
Hm86 (Fra)	19/382 (4.97)	14/127 (11.08)	19/382 (4.97)	14/127 (11.08)	19/382 (4.97)	14/127 (11.08)
Hd86-A1 (Tun)	23/382 (6.02)	13/127 (10.24)	23/382 (6.02)	13/127 (10.24)	23/382 (6.02)	13/127 (10.24)
Bm86 (Moz)	105/382 (27.48)	43/127 (33.86)	105/382 (27.48)	43/127 (33.86)	105/382 (27.48)	43/127 (33.86)
Bm95 (Arg)	105/382 (27.48)	43/127 (33.86)	105/382 (27.48)	43/127 (33.86)	105/382 (27.48)	43/127 (33.86)
Bm86 (Aus)	106/382 (27.75)	44/127 (34.65)	106/382 (27.75)	44/127 (34.65)	106/382 (27.75)	44/127 (34.65)

Note: *H. excavatum*: He86-A1/A2/A3 (Tun) (Ariana, Tunisia; HQ992990, HQ992991 and HQ992992, respectively; present study), He86-S (Tun) (Sousse, Tunisia; JF298786 [33].
Hyalomma anatolicum: Ha98 (Ind) (Ludhiana, India, AF347079 [26], Haa86 (Iza) (Izatnagar, India, EU665682 [35].
H. m. marginatum: Hm86 (Fra) (Corsica, France, GU144608 [34] *H. scupense*: Hd86-A1 (Tun) (Ariana, Tunisia; HQ872020 [28].
R. microplus: Bm86 (Aus) (Yeerongpilly-Australia, M29321 [23], Bm86 (Moz) (Mozambique, EU191620 [34], Bm95 (Arg) (Corrientes Province, Argentina, AF150891 [37].

Table 1: Nucleotide and amino acid comparison of analysed *Hyalomma excavatum* Bm86 partial sequences and their sequence orthologs from *Rhipicephalus* genus and other *Hyalomma* species.

	He86-S integral sequence[1]		He86-A1/A2/A3 partial sequences[2]	
	Nucleotide diversity (%)	AA diversity (%)	Nucleotide diversity (%)	AA diversity (%)
Bm86 (Aus)	26	34	27	34
Bm95 (Arg)	26	35	27	33
Bm86 (Moz)	29	40	27	33
Hd86-A1 (Tun)	8	12	6	10

Note:
[1]The size of the He86-S integral nucleotide sequence without the signal peptide is 1833 pb giving a putative protein of 611 aa.
[2]The size of the analysed Hsp86-A partial nucleotide sequence is 382 pb giving a putative protein fragment of 127 aa.

H. sp.: Hsp86-A1 (Tun), Hsp86-A2 (Tun) and Hsp86-A3 (Tun) (Ariana, Tunisia; Genbank accession number HQ992990, HQ992991, HQ992992, respectively); *H. scupense*: Hd86-A1 (Tun) (Ariana, Tunisia; HQ872020) [28]; *H. excavatum*: He86-S (Tun) (Sousse, Tunisia, JF298786 [33]; *R. microplus*: Bm86 (Aus) (Yeerongpilly-Australia, M29321) [23], Bm86 (moz) (Mozambique, EU191620) [34], Bm95 (Arg) (Corrientes Province, Argentina, AF150891) [37].

Table 2: Nucleotide and amino acid diversity of integral He86-S and partial He86-A1/A2/A3 sequences compared to vaccine orthologs from *Rhipicephalus* (*Boophilus*) *microplus* and *Hyalomma scupense*.

Position[b]	Hd86(A1)	He86(A1)	He86(A2)	He86(A3)
23 (8)	G (G)	G (G)	G (G)	T (V)
27 (9)	G (K)	A (K)	A (K)	A (K)
33 (11)	C (F)	T (F)	T (F)	T (F)
39 (13)	A (Q)	G (Q)	G (Q)	G (Q)
41 (14)	G (S)	A (N)	A (N)	A (N)
130 (44)	A (K)	C (Q)	C (Q)	C (Q)
138 (46)	G (E)	A (E)	A (E)	A (E)
171 (57)	T (S)	C (S)	C (S)	C (S)
177 (59)	G (G)	A (G)	A (G)	A (G)
178 (60)	C (H)	A (N)	A (N)	A (N)
184 (62)	A (I)	G (V)	G (V)	G (V)
187 (63)	G (E)	C (Q)	C (Q)	C (Q)
190 (64)	G (V)	A (I)	A (I)	A (I)
193 (65)	G (G)	A (S)	A (S)	A (S)
209 (70)	G (G)	A (D)	A (D)	A (D)
249 (83)	T (C)	C (C)	C (C)	C (C)
266 (89)	T (A)	A (A)	A (A)	A (A)
305 (102)	T (L)	G (R)	G (R)	G (R)
324 (108)	T (L)	C (L)	C (L)	C (L)
327 (109)	T (G)	C (G)	C (G)	C (G)
328 (110)	G (A)	C (P)	G (A)	G (A)
335 (112)	G (C)	G (C)	A (Y)	G (C)
357 (119)	A (K)	G (K)	G (K)	G (K)
362 (121)	A (N)	G (S)	G (S)	G (S)
368 (123)	C (A)	G (G)	G (G)	G (G)

Note: [a]Amino acids generated by each substituted nucleotide are shown between parentheses.

[b]Nucleotide and amino acid positions are referred to Hd86-A1 nucleotide and amino-acid sequences, GenBank accession number HQ872020.

Table 3: Nucleotide and amino acid[a] substitutions between He86(A1/A2/A3) and Hd86(A1) sequences

and coordination and drafted the manuscript. All authors read and approved the final manuscript.

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