3'-RACE Amplification of Aminopeptidase N Gene from *Anopheles stephensi* Applicable in Transmission Blocking Vaccines

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Abstract

Background: Because of the lack of an effective and economical control strategy against malaria (the most devastating infectious disease in developing countries) Transmission-Blocking Vaccines (TBVs) concept has been raised in recent years, promising a more efficient way to malaria control. TBVs aim at interfering and/or blocking pathogen development within the vector, halting transmission to non-infected vertebrate host. Aminopeptidase N (APN) is one of the most potent proteins in parasite development in Anopheles malaria vectors, which is strongly co-localized with human malaria parasites in the mosquito midgut epithelium. Therefore, Aminopeptidase N is one of the best choices for a new TBV.

Methods: In this study for the first time we used 3'-RACE to amplify APN gene in *Anopheles stephensi* (*An.stephensi*), a major malaria vector in Iran, Indian subcontinent up to China by using different sets of primers including exon junction, conserved and specific region primers.

Results: Full length of APN was sequenced stepwise, which could be applied in designing a new regional TBV and act as an essential component of malaria elimination program in *An.stephensi* distribution areas.

Conclusion: Primers design and method modification should be set up exactly in approach based amplifications. From results we came to this conclusion that that 3'-RACE could be applied to amplified key regions which are beyond reach.

Introduction

Malaria is one of the most serious infectious diseases. It is caused by protozoan parasites of the genus *Plasmodium* and transmitted to humans through the mosquito's bites of the genus *Anopheles* [1]. In humans, the most severe form of malaria is caused by *Plasmodium falciparum* (*P.falciparum*), and at least one-third of the world's population is at the risk of infection, with over 300 million people developing clinical disease and at least 2 million deaths each year [2]. Other *Plasmodium* spp. including *Plasmodium vivax* (*P.vivax*) infect humans and cause considerable morbidity in endemic populations. Major vector of Iran is *An.stephensi* [3]. While there have been major reductions in mortality and morbidity in some areas such as South Asia, malaria remains a major pediatric killer in many parts of sub-Saharan Africa, which bears the greatest burden of disease [4].

There is lack of a cost-effective control strategy against malaria. The measurement of global eradication based upon the use of insecticides and chemotherapy has greatly reduced its incidence but administrative and financial problems, aggravated by the spread of insecticide resistant mosquitoes. Drug resistant parasites have reversed the gains in some areas, particularly in South East Asia [5].

To be passed on to humans, the parasite requires successful completion of the sporogonic cycle in the midgut and the salivary glands of the mosquito [6]. The female mosquito is responsible for blood digestion and is the first host for interaction with the parasite [7]. *Plasmodium* must complete its development in the *Anopheles* mosquito before transmission to the new host [8,9]. *Plasmodium* ookinetes form in the mosquito's midgut luminal blood meal and migrate to the periphery where is thought to recognize midgut ligands [10]. Recognition is followed by cell invasion and differentiation into oocysts between the midgut basal cell surface and the basal lamina. Each oocyst releases thousands of sporozoites that invade the mosquito salivary glands and are delivered to a vertebrate host during a succeeding blood meal [11].

Clearly, the ookinete-to-oocyst transition is crucial for successful parasite establishment in the mosquito and therefore, represents the best paradigm to develop novel interventions [12]. One promising approach is the use of anti-vector malaria Transmission-Blocking Vaccines (TBVs). It prevents ookinete-to-oocyst transition by targeting mosquito's midgut ligands that mediate parasite cell adhesion as opposed to classical TBVs that target surface molecules on parasite sexual stages [13,14]. Unlike classical vaccine approaches, TBVs do not protect the vaccinated individual from contracting malaria but are intended to prevent parasite development in the mosquito, and thereby limit the number of infectious vectors [15,16].

For a molecule to be an effective TBV candidate, certain basic principles must be followed. First, it has to induce high antibody titers in order to block pathogen development within the insect completely [9]. Additionally, in case the TBV candidate is presented in an antigen/adjuvant combined ion, this combination has to be safe enough to the vertebrate host in order to prevent significant side effects following immunization [17]. Previously, *P. falciparum* proteins Pfs25, Pfs28, Pfs48/45 and Pfs230 and their orthologs in *P.vivax* were tested in transmission-blocking assays [18-21]. In the context of malaria transmission, recent evidence suggests that *Plasmodium* parasites use multiple mosquito midgut molecules as adhesion ligands, which include glycans (carbohydrates) [22-24] and enzymes such as alanyl aminopeptidase (APN).

Previous studies have shown when rabbit polyclonal antibodies were directed against the N-terminal portion of APN passively transferred to *Plasmodium berghei* (*P.berghei*) infected mice, they were able to significantly reduce the number of oocysts in both *An.gambiae* and *An.stephensi* [25]. Aminopeptidase N belongs to a group of membrane-bound zinc enzymes [26]. It is a ubiquitous enzyme which is found in a wide range of organisms from insects to mammals. In insects, several APNs have also been identified and cloned from the gut epithelial cells of various species [27].

In this study, we characterized *An.stephensi* APN by using 3'-RACE technique to produce an efficient vaccine against sexual stage of *Plasmodium* spp within *An.stephensi*, which is the most important vector of Iran, China and eastern Mediterranean region. For this reason, extracted RNA was used to synthesize the first strand cDNA. Then Reverse Transcriptase enzyme according to RT-PCR kit was used by the linker primer which composed of inner, outer and oligo (dT) at the 3'-end to amplify the desired sequence.

Thus, in this study not only using 3'-RACE technique, the ending sequence of APN gene has been amplified, but also, amplification and sequencing of mid-region is performed. For better understanding of sequence importance, motif prediction, phylogenetic tree, anti-

genicity and description of protein active site are provided too. The obtained results proved its usefulness in further application of this part of genome for designing a regional TBV.

Materials and Methods

Mosquito samples

An.stephensi samples were collected by total catch and hand catch collection methods during 2009-2011 from the malaria endemic areas of Iran; Iranshahr, Chabahar, Khash, Zabol, Zahedan, Sarbaz, Saravan, and Nikshahr districts, which are located in Sistan and Baluchistan province, south-east of Iran.

RNA extraction

Total RNA was extracted from female's An.stephensi tissue by QIAZOL according to manufacture's instruction. RNA was precipitated and solubilized in DEPC (Diethyl pyrocarbonate) treated water and then stored in -70^oC.

cDNA synthesis

Extracted RNA was used for the first strand cDNA synthesis. Then RT (Reverse Transcription) reaction according to RT-PCR kit (Fermentas) was performed by the linker primer (5'GAGATTTGAATCTTGCTTCTGGG CCCTCTATTGTCATTGTCTTTTTTTTTTT TTTTTT-3') that had been composed of inner (5'-GGCCCTCTATTGTCATTGTC-3'), outer (5'-AGATTTGAATCTTGCTTCTG-3') and oligo (d.T) at the 3'-end.

Exon junction primer designing

Four exon junction primers (F1: 5'-TGTCG CCTATGACAAATCTG-3', F2: 5'-GTGGAG GAAATGAATAAGCTC-3', F3: 5'-CAATC TGGACTGGGTGAATG-3', F4: 5'-GACTAC GGTGACTGCAGGAC-3') were designed by Oligo6 software (version 6.54, 2001) and BLAST (online tool) as forward primers of Aminopeptidase N gene according to its resemble gene in An.gambiae with XM_ 318000.4 accession number in the GenBank.

Polymerase chain reaction

cDNA was used as a template in subsequent PCR reactions with Taq DNA polymerase. The desired region was amplified by exon junction primers as a forward and 3' outer primer as a reverse primer for performing PCR to amplify the 3'-end of the APN gene. The reaction was run for 35 cycles in a GeneAmp PCR System 2400 (Perkin, Elmer), with the following cycle temperatures and times: 94^oC, 5 min; 94^oC, 1 min; 60^oC, 1 min; 72^oC, 1 min plus 10 min extra extension time in the last cycle. Confirmation PCR was done using exon junction primers as forward and 3'-inner as a reverse primer. PCR product was run on agarose gel, then desired band was purified with PCR product purification kit (Promega).

Cloning

The confirmed PCR product was TA cloned into the pDrive vector using the PCR Cloning Plus kit (Qiagen, Hilden, Germany) according to manufacturer's instruction. In this way, the ligation reaction was prepared by mixing 2X Rapid Ligation Buffer, pDrive Vector and T4 DNA Ligase with purified PCR product as an Insert DNA. After overnight incubation at $4°C$, the ligation product was transformed into competent Escherichia coli (E.coli) M15 and then incubated overnight at $37°C$. So, white clones which include insert were transferred to LB broth and incubate overnight at 37^oC in shaker incubator. Plasmids were extracted using QIAGEN plasmid extraction kit (Germany). Extracted plasmids were run on the 0.8% agarose gel. Recombinant vectors have higher weigh and are selected for further analysis. After confirmation by digestion and colony PCR, sequencing analysis was performed by Chromas (Version 2.31, 2005), DNA star (Version 7.10, 2006), MEGA5 (Build 5110426, 2011) and BLAST.

Conserve primer designing

Six primers (1F: 5'-GGCTACTATCGCGT CAACTAC-3', 2F: 5'-GAATACATCTGGC TGAACGA-3', 3F: 5'-AGTACGCTCACCA GTGGTT-3', 2R: 5'-GCGCAGACCGTAAC AGTAC-3') were designed based on conserved regions of Aminopeptidase N gene in An.gambiae. The desired region was amp-

lified by conserved primers. PCR cycle temperatures and times applied similar to exon junction amplification. 2X Rapid Ligation Buffer, pGEM-T Easy Vector and T4 DNA Ligase were used for ligation reaction. Purified PCR product was used as an Insert DNA again. Confirmation of insertion in plasmid was done by colony PCR and the final step was sequencing.

Specific primers

Forward primers (F2116: 5;-CATCCTATT CGTGGACGC-3', F2143: 5'-TCGAAGAAC TGTACGGACTGT-3', F2353: 5'-GCGCAA TCACGAATTCTG-3', F2518: 5'-CACAAC ATACGAGCCGGA-3') were designed from the specific region of APN in *An.stephensi* that achieved in the previous step. During PCR, 3'-end region of APN was amplified by specific primers (as forward) and 3'-outer primer (as a reverse) in separate reactions. Confirmation was done by specific and 3'inner primers again. PCR conditions repeated exactly as above. Cloning was done and analysis of sequences revealed the quality of results.

Results

After RNA extraction from the whole body of female mosquito, cDNA was synthesized with linker primer during reverse transcription reaction. Then according to our previous studies on *An.stephensi* and its high genomic similarity with *An.gambiae* we decided to use exon junction primers for 3'-RACE to increase the specificity of the primers. By using exon junction designed primer (F4) from APN gene in *An.gambiae* as a forward and outer primer as a reverse primer, a region with 516 *bp* at 3'-end of mRNA has been amplified (Figure 1). This was followed by recovery of amplified product from agarose gel that was confirmed by using the inner primer as a reverse primer according to 3'-RACE. Sequencing result showed that the cloned fragment was not related to 3'-end of APN. It showed that the APN gene of *An.gambiae* and *An.stephensi* are not very similar. This was a step forward toward amplification of APN gene as it showed that the use of 3'-RACE along with exon junction primers is not a proper way to amplify APN gene.

Global alignment of the deduced sequence by using MegAlign for APN gene (XM_318000.4) showed no similarity with the expected target sequence of APN. So, it has been decided to align APN gene of different strains to design new primers for conserved regions of APN gene. After designing the primers from conserved sequence of APN in *An.gambiae*, we amplified 513 *bp* sequence with 1F and 2R primers, 1182 *bp* sequence with 2F and 2R primers and 1229 *bp* sequence with 3F and 2R primers (Figure 2). These sequences had overlaps and they covered the mid-region of APN. After analy-
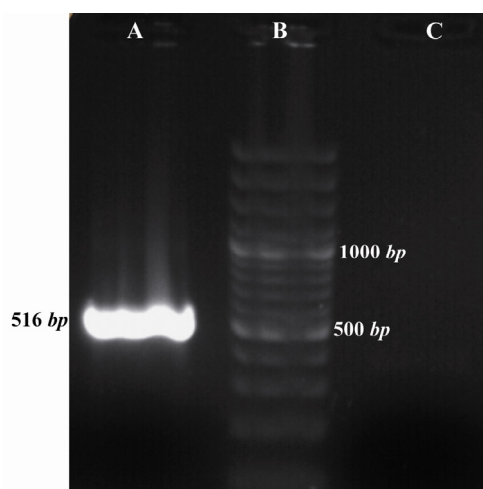

Figure 1. Amplification of APN using exon junction primers. A) 516 *bp* fragment amplified with exon junction primers, B) 100 *bp* DNA Ladder, C) Negative control
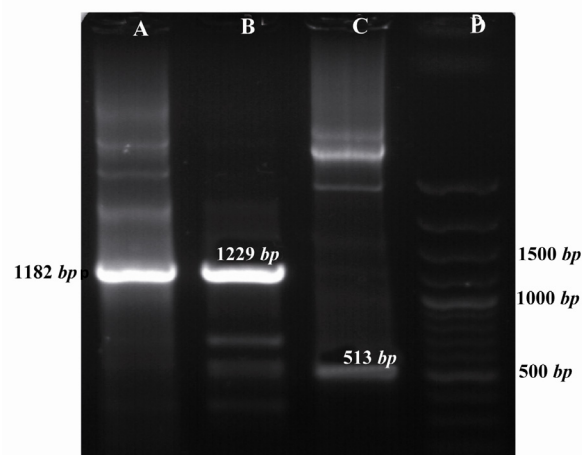

Figure 2. PCR Amplification of APN in *Anopheles stephensi* using conserved primers. A) 1182 *bp*, B) 1229 *bp*, C) 513 *bp* sequences covering the mid-region of APN, D) 100 *bp* DNA ladder

Query 91   CAGATTGGCTACTATCGCGTCAACTACGACGATCGCAACTGGGAGCTCATCACGAACGCG 150
           ||| | |||||||||||||||||||||||||||| | | |||||||||||||| || ||||||
Subject 1838 CAGGTCGGCTACTATCGCGTCAACTACGACGAACACAACTGGGAGCTCATTACTAACGCG1897

Query 151 CTGATCGACACCTTG-CCGCCGTGCACCGGCTCAACCGGGCGCAGCTGATCGATGACGCC 209
           || ||| || || | || || | | ||||| ||||| |||||||| ||||||||||
Subject 1898 CTACACGAGAACTGGGCCAGCATACATCGGCTTAACCGAGCGCAGCTCATCGATGACGCC1957

Query 210 TACTGGCTGGCACGGTCCGGCCGCCTTGACCTGCGCGTCGCCCTCCGCCTGATGACGTAC 269
           ||||||| |||| |||| ||||| ||||| | | || ||||| || | | |||||||||||
Subject 1958 TACTGGTTGGCTCGATCCGGCCGGCTCGATCTGCGCGTAGCCCTGCGTTTCATGACGTAC2017

Query 270 CTGCGCGGTGAGCGGGAGTATGCGCCGTGGGCGGCAGCGAACACTGCCCTGTCCTACTTC 329
          |||||| |||||||||||||| || |||||| |||||| || ||| |||| |||||||||
Subject 2018CTGCGCAACGAGCGGGAGTACGCACCGTGGACGGCGGCAAACGTTGCCCTAACTTACTTC 2077

Query 330 AACAGCCGTCTCCGCGGTACGGAGAATTACCACGATTTCATCCTATTCGTGGACGCTCTG 389
          |||| ||||||||||||| |||| | |||||| | |||| || |||||||| || |||
Subject 2078AACAACCGTCTCCGCGGAACGGCCGAGTACCACAACTTTTTGATATTCGTGGATGCGCTG 2137

Query 390 ATCGAAGAACTGTACGGACTGTTGACGATCGACGTGGTATCGCCGAACGATTCACTGCTG 449
          ||||||| | | |||| | || || | ||||||||||| || |||| | | ||| ||
Subject 2138ATCGAAGACATCTACAGTTTGCTAACGATCGACGCAGTTTCGCCCGACGACACGCTGTTG 2197

Query 450 CACAAGTACCTCGTGCAGACGATCACCAGCTGGGCCTGCTCGTTGGGCTACAAGGACTGT 509
          ||||||| | ||||||||||| | | ||||||| |||||||| ||||| |||| ||||||
Subject 2198CACAAGTATCTCGTGCAGACTATTTCCACCTGGGCTTGCTCGATGGGTTACACTGACTGT 2257

Query 510 CTGGAGCGGACGGGCGCCCTGCTGAGGGCCGGGGCATCGGGCACGGGACCAGCCGTACAT 569
          ||| | ||||| ||| ||| ||| || ||| | || || ||||||||||||||||| |||
Subject 2258CTGATGAAGACGGCCGCTCTGTTGAAGGCTGAAGCTAGTGGGACGGGTCCAGCCGTCCAT 2317

Query 570 CCCGACATTGCCACCGTCACGTACTGTTACGGTCTGCGC 608
          || ||||||||||| ||| ||||||||||||| |||||
Subject 2318CCGGACATTGCCAGCGTGACGTACTGTTACGGTATGCGC 2356

Figure 3. Global alignment of amplified APN mid-region in *Anopheles stephensi* with APN sequence in *An.gambiae* (XM-3180000)

sis of the cloning results, we achieved the mid region of APN in *An.stephensi*.

Global alignment of mid-region of APN gene in the GenBank is shown below (Figure 3). It has the maximum identity to XM_ 001689265.1 (*An.gambiae* str. PEST AGAP 001881-PA) and XM_318000.4 (*An.gambiae* str. PEST AGAP001881-PA) by maximum identity of 78% and 74% respectively.

At last step toward amplifying overall sequences of APN we designed specific primers according to the mid-region of APN in *An. stephensi* which was sequenced by conserved primers. Here, we reached the 1102 *bp* at 3'-end sequence of APN by F2143 specific and 3'outer primer (Figure 4).
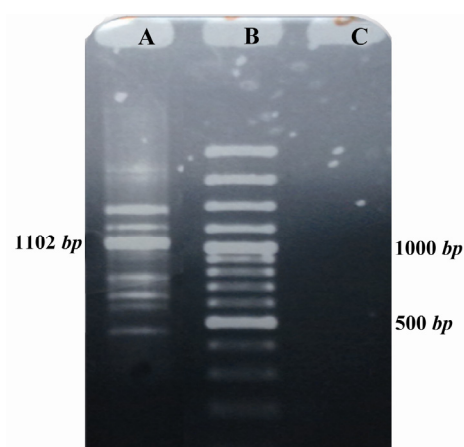

Figure 4. Amplification of APN in *Anopheles stephensi* using specific primers. A) 1102 *bp* amplified mid-region of APN, B) GeneRuler™ 100 *bp* plus DNA ladder, C) Negative control

Alignment of new amplified sequence in the GenBank showed increased identity in comparison with previous alignment. Here, we found that our sequence has the most similarity to EU827825.1 (*An.gambiae* strain G3 Aminopeptidase mRNA).

Eventually, we reported partial sequences of APN which was the active site of APN in *An.stephensi.* So, we would be able to use this sequence as a very important base of anti-APN antibody production.

A phylogenetic tree was constructed based on the APN amino acid sequence of different insects by the Maximum Likelihood method (Figure 5). In this method, an initial tree is first build using a fast but suboptimal method such as Neighbor-Joining, and its branch lengths are adjusted to maximize the likelihood of the data set for that tree topology under the desired model of evolution. Then variants of the topology are created using the NNI (nearest neighbor Interchange) method to search for topologies that fit the data better. Maximum-likelihood branch lengths are com-
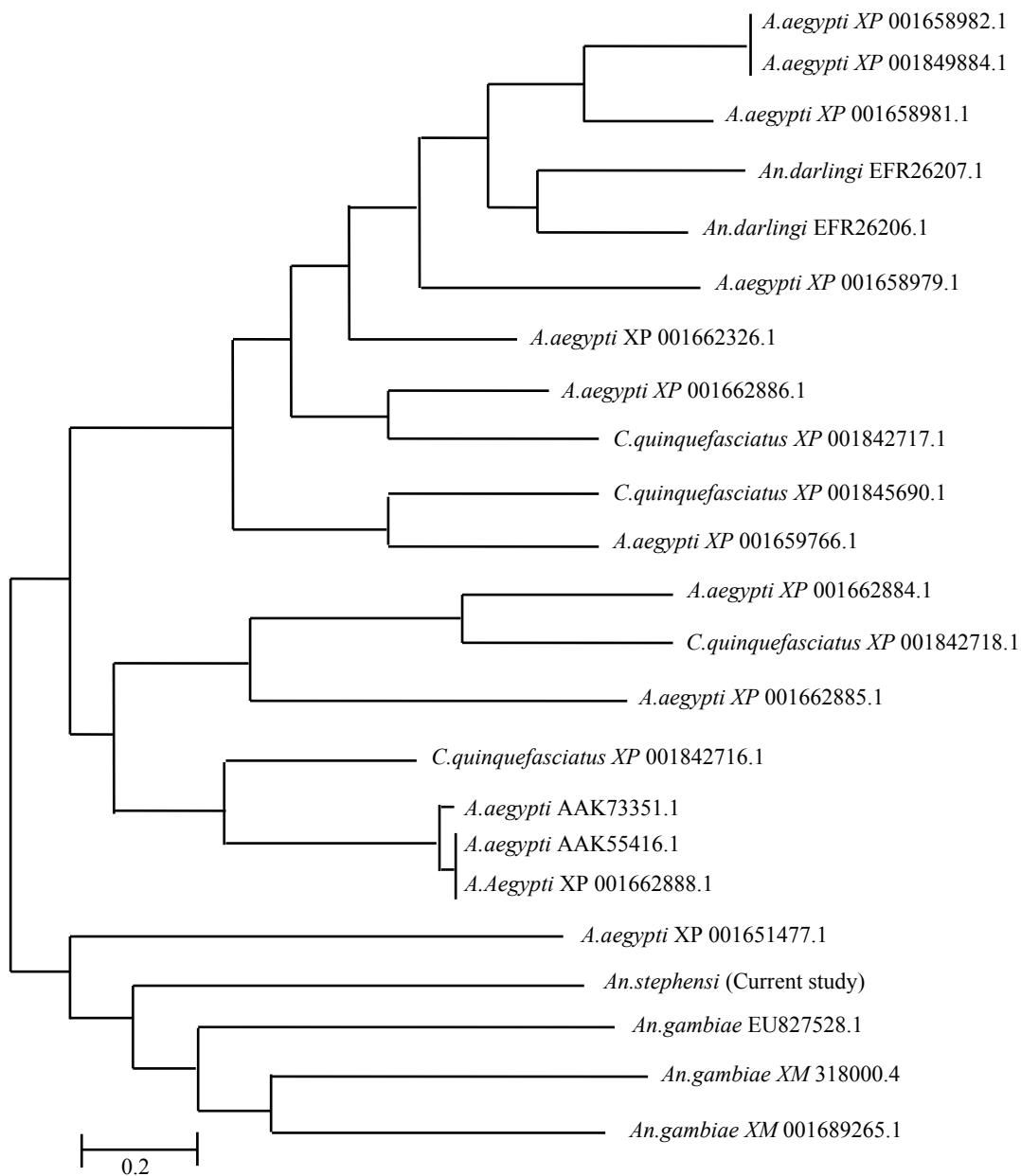
Figure 5. Phylogenetic tree constructed based on amino acid sequence of APN in *Anopheles stephensi* and other APN-related sequences in GenBank by Maximum Likelihood method using Mega5 software

puted for these variant tree topologies and the greatest likelihood retained as the best choice so far. This search continues until no greater likelihoods are found. Phylogenetic has constructed by Mega5 software (build#5110426). Our sequence had most similarity to sequences XP001651477.1 and EU827825.1. This result is in accordance to nucleotide analysis that maximum identity was related to EU827825.1 (*An.gambiae* strain G3 Aminopeptidase mRNA).

Discussion

Obviously, there has been lack of a cost-effective and applicable control strategy against malaria. The ookinete-to-oocyst transition is crucial for successful parasite establishment in the mosquito and therefore, represents the best paradigm to develop novel interventions. One promising approach is the use of anti-vector malaria TBVs that prevent ookinete-to-oocyst transition by targeting mosquito midgut ligands that mediate parasite cell adhesion opposed to classical TBVs, which target surface molecules on parasite sexual stages [28].

As their name suggests, TBVs target the parasite stages that develop within the mosquito vector, and as a result, prevent the subsequent cascade of events that leads to infection of human hosts. All malaria vaccine approaches are intended to introduce herd immunity in the target community. Unlike classical vaccine approaches, TBVs do not protect the vaccinated individual from contracting malaria but are intended to prevent parasite development in the mosquito, and thereby limit the number of infectious vectors. Consequently, TBVs have been categorized as 'altruistic vaccines'. Two main paradigms can be used in the design of TBVs: They can target either parasite or mosquito antigens. The ultimate goal for examining potential mosquito midgut antigens is to develop a global TBV that works against all human malaria parasites across different *Anopheline* species. Malaria parasites must undergo development within mosquitoes to be transmit-

ted to a new host. Anti-vector transmission-blocking vaccines inhibit parasite development by preventing ookinete Interaction with mosquito midgut ligands. Therefore, the discovery of novel midgut antigen targets is paramount. Unfortunately, progress has been too slow and transmission-blocking mosquito antigens have not been characterized. Aminopeptidase N is a conserved and putative ligand for both murine and human *Plasmodium* ookinetes in diverse mosquito vectors [29].

Jacalin (a lectin) inhibits ookinete attachment by masking glycan ligands on midgut epithelial surface glycoprotein's. Aminopeptidase N is the predominant Jacalin target on the mosquito midgut luminal surface and provides evidence for its role in ookinete invasion. Inhibition of Aminopeptidase N strongly blocked both *P.berghei* and *P.falciparum* development in different mosquito species, implying that Aminopeptidase N has a conserved role in ookinete invasion in the midgut. Also attempts to disrupt the Aminopeptidase N gene, which suggests that the enzyme makes an important contribution in hemoglobin catabolism during the intra erythrocytic cycle [30].

In this study, attempts were focused on amplification of APN gene in *An.stephensi* using 3'-RACE for the first time. Rapid Amplification of cDNA Ends (RACE) was used to obtain the full length sequence of RNA transcript. So, RACE resulted in production of a cDNA copy of the APN RNA sequence, produced through reverse transcription and followed by PCR amplification of the cDNA copies. The amplified cDNA copies were sequenced and used to map to a unique APN mRNA and almost its full sequence is known.

The first step in our study was to use reverse transcription to produce cDNA copy from a region of the RNA transcript. In this process, 3'-end portion of APN is copied using a known sequence from the center of the transcript. The copied region is bounded by the known sequence, and 3'-end. Here exon junction primer was not good for amplification of the APN mid region gene. So, the

conserved primer was used. By using conserved primers the mid region of APN was amplified and then it was used to design a new set of primers called Gene Specific Primer (GSP). Our gene specific primers recognized a known sequence in the gene of APN and they copied the mRNA template in the 5' to 3' direction to generate a specific single-stranded cDNA product of APN gene. There are some other ways to add the 3'-terminal sequence for the first strand of the de novo cDNA synthesized which are much more efficient than homopolymeric tailing and they can be used by other scientist, but the sense of the method remains the same. Finally, we used a PCR reaction which used a second anti-sense gene specific primer (GSP2) that binds to the known sequence, and a sense (forward) universal primer (UP) that binds the homopolymeric tail added to the 3' ends of the cDNA to amplify a cDNA product from the end of APN sequence.

Amplification of APN gene has been followed by evaluation of its amino acid sequence for active site prediction. Homology modeling of APN has shown that APN has five domains: Domain I is the cytosolic part of APN, which contains nine residues [31]. Domain II is the membrane-spanning domain probably existing as one a-helix. Domain III has a stalk region between residue 40 and residue 70, and after this residue there is a sequence WNXXRLP in APN, which is homologous to non-membrane bound aminopeptidases [32]. Domain IV is composed of amino acid residue 70 to 252 and residues 216-227 is

Prosite pattern					
Found Motif	Position	Prosite	Description	Related Sequences	Related structures
EGF_1					
	9..20 380..391 505..516 596..607 632..643 Delail 851..862 897..908 1043..1054 1224..1235	PS00022	EGF-Ske domain signahare 1	701	--
VWFC_1					
	211..258 214..258 500..551 505..551 Delail 577..623 684..735 1039..1092	PS01208	VWFC domain signahare	120	--
4FE4S_FER_1					
	391..402 Delail	PS01208	4Fe-4S ferredoxin-type iron-sulfur binding region signahare	1326	--
2FE2S_FER_1					
	27..35 358.366 Delail 1114..1122	PS00197	2Fe-25 ferredoxin-type iron-sulfur binding region signahare	239	--

Figure 6. Analysis of deduced amino acid sequence of APN in *Anopheles stephensi* for motif predication that found 4 motifs in assigned positions. Number of found motifs: 4

Table 1. Antigenicity and epitope prediction of APN amplified sequence in *Anopheles stephensi*

Peptide length 10		MHC I					
		Syfpeithi					
Pos	Sequence	A*0301	A*1101	A*2402	A*2601	A*6801	B*3801
694	CTGGCTGTCR	10.0	19.0	0.0	9.0	22.0	2.0
757	TMACAAGCAK	15.0	17.0	1.0	3.0	10.0	1.0
795	ATCGCCGCAR	13.0	22.0	0.0	10.0	18.0	1.0

a conserved region that is indicated to be related to the APN enzymatic activity. Domain V and domain VI include amino acids 253-580 corresponding to exons 3 to 10 [33]. There locates a glutamic acid 355 (E355) in APN conserved region and the mutation of this residue induce a large decrease in enzyme activity, even led to an almost completely inactive enzyme when it is mutated to A [34]. Domain VII constitutes the remaining C-terminal part of the enzyme and includes amino acids 581 to 967 (exons 11- 20). This domain has a very high content of predicted α-helices [35]. Active site of the enzyme is buried in the middle of the protein.

The results of this study showed that a single arginine is essential for enzymatic activity, and more precisely for substrate binding in Aminopeptidase N. There are two openings to the active site cavity. The first opening (N-terminal channel) comprises a shallow 8-Å- long groove at the junction of domains I and IV. The second and larger opening (C-terminal channel) is formed by the C-terminal domain IV, which comprises 8 pairs of -helices arranged in 2 layers to form a cone-shaped super helical structure. This domain interacts with the catalytic domain II and contains a 30-Å-long channel leading towards the active site [36].

To evaluate the amplified sequence in vaccine design one of the most important thing for analysis is the number of motifs or signatures in that sequence. Using motif finding shareware (http://myhits.isb-sib.ch/cgi-bin/motif_scan) the amplified sequence was analyzed. Here, we found 4 motifs. Two first founded motif, EGF_1 and VWFC_1 are common motifs of aminopeptidases (Figure 6).

Antigenicity is the ability of a chemical structure (referred to as an antigen) to bind specifically with certain products of adaptive immunity: T cell receptors or antibodies. Thus, an antigen might bind specifically to a T or B cell receptor, but not induce an adaptive immune response. Antigenicity could be predicted using Hopp and Woods hydrophilicity method for locating antigenic determinants. Here, by the use of ETK epitoolkit online tools (http://www. epitoolkit.org/epipred/predictions), our sequences have been evaluated from the point of antigenicity. Result has shown that three distinct parts of our protein can be used as an epitope (Table 1), including position 694: CTGGCTGTCR, 757:TMACAAGCAK, and 795: ATCGCCG CAR.

Conclusion

This method has resulted in amplification of APN gene for the first time from *An.stephensi* that is the most important malaria vector of Iran, Indian sub-continent up to China. At present time APN as well as few other molecules, like Sagalin proteins is the primary candidate in production of transmission blocking vaccines. The obtained results from the current project seems to provide the prerequisite genomic data on the structure of APN in a major malaria vector that its anti-APN challenge will promote the designing of a regional TBV, an essential component for malaria elimination and final stage of eradication in Iran and neighboring countries.

Acknowledgement

References

1. Frischknecht F, Martin B, Thiery I, Bourgouin C, Menard R. Using green fluorescent malaria parasites to screen for permissive vector mosquitoes. Malaria J 2006;5:23-29.

2. Snow RW, Guerra CA, Noor AM, Myint HY, Hay SI. The global distribution of clinical episodes of Plasmodium falciparum malaria. Nature 2005;434 (7030):214-217.

3. Greenwood BM, Bojan K, Whitty CJ, Targett GA. Malaria. Lancet 2005;365(9469):1487-1498.

4. Bosman A, Mendis KN. A major transition in malaria treatment: the adoption and deployment of artemisinin-based combination therapies. Am J Trop Med Hyg 2007;77(6 Suppl):193-197.

5. Collins WE, Jeffery GM. Plasmodium malariae: parasite and disease. Clin Microbiol Rev 2007;20 (4):579-592.

6. Rodrigues J, Agrawal N, Sharma A, Malhotra P, Adak T, Chauhan VS, et al. Transcriptional analysis of an immune-responsive serine protease from Indian malarial vector, Anopheles culicifacies. BMC Mol Biol 2007;8:33.

7. Xu WY, Huang FS, Hao HX, Duan JH, Qiu ZW. Two serine protease from Anopheles dirus heamocytes exhibit changes in transcript abundance after infection of an incompatible rodent malaria parasite, Plasmodium yoelii. Vet Parasit 2006;139(1-3): 93-101.

8. Barillas-Mury C, Kumar S. Plasmodium–mosquito interactions: a tale of dangerous liaisons. Cell Microbiol 2005;7(11):1539-1545.

9. Ghosh A, Edwards MJ, Jacobs-Lorena M. The journey of the malaria parasite in the mosquito: hopes for the new century. Parasitol Today 2000;16 (5):196-201.

10. Han YS, Thompson J, Kafatos FC, Barillas-Mury C. Molecular interactions between Anopheles stephensi midgut cells and Plasmodium berghei: the time bomb theory of ookinete invasion of mosquitoes. EMBO J 2000;19(22):6030-6040.

11. Tsuboi T, Cao YM, Hitsumoto Y, Yanagi T, Kanbara H, Torii M. Two antigens on zygotes and ookinetes of Plasmodium yoelii and Plasmodium berghei that are distinct targets of transmission-blocking immunity. Infect Immun 1997;65(6):2260-2264.

12. Malkin EM, Durbin AP, Diemert DJ, Sattabongkot J, Wu Y, Miura K, et al. Phase 1 vaccine trial of Pvs25H: a transmission blocking vaccine for Plasmodium vivax malaria. Vaccine 2005;23(24): 3131-3138.

13. Kaslow DC, Shiloach J. Production, purification and immunogenicity of a malaria transmission-blocking vaccine candidate: TBV25H expressed in yeast and purified using nickel-NTA agarose. Biotechnology 1994;12(5):494-499.

14. Kumar N, Ploton I, Koski G, Ann-Lobo C, Contreras C. Malaria transmission-blocking immunity. Identification of epitopes and evaluation of immunogenicity. Adv Exp Med Biol 1995;383:65-72.

15. Saul A. Minimal efficacy requirements for malarial vaccines to significantly lower transmission in epidemic or seasonal malaria. Acta Trop 1993;52(4): 283-296.

16. WHO. Malaria transmission-blocking vaccines: an ideal public good. WHO document no. (TDRyRB MyMALyVACy2000.1) available from WHO, Geneva, and on website available from http:www.who.intytdr 2000.

17. Quakyi IA, Carter R, Rener J, Kumar N, Good MF, Miller LH. The 230-kDa gamete surface protein of Plasmodium falciparum is also a target for transmission-blocking antibodies. J Immunol 1987;139 (12):4213-4217.

18. Duffy PE, Kaslow DC. A novel malaria protein, Pfs 28 and Pfs25 are genetically linked and synergistic as falciparum malaria transmission-blocking vaccines. Infect Immun 1997;65(3):1109-1113.

19. Hisaeda H, Stowers AW, Tsuboi T, Collins WE, Sattabongkot JS, Suwanabun N, et al. Antibodies to malaria vaccine candidates Pvs25 and Pvs28 completely block the ability of Plasmodium vivax to infect mosquitoes. Infect Immun 2000;68(12): 6618-6623.

20. Sattabongkot J, Tsuboi T, Hisaeda H, Tachibana M, Suwanabun N, Rungruang T, et al. Blocking of transmission to mosquitoes by antibody to Plasmodium vivax malaria vaccine candidates Pvs25 and Pvs28 despite antigenic polymorphism in field isolates. Am J Trop Med Hyg 2003;69 (5):536-541.

21. Rosenberg R, Koontz LC, Alston K, Friedman FK. Plasmodium gallinaceun: erythrocyte factor essential for zygote infection of Aedes aegypti. Exp Parasitol 1984;57 (2):158-164.

22. Dinglasan RR, Fields I, Shahabuddin M, Azad AF, Sacci JB Jr. Monoclonal antibody MG96 completely blocks Plasmodium yoelii development in Anopheles stephensi. Infect Immun 2003;71(12):6995-7001.

23. Dinglasan RR, Valenzuela JG, Azad AF. Sugar epi-topes as potential universal disease transmission blocking targets. Insect Biochem Mol Biol 2005;35 (1):1-10.

24. Dinglasan RR, Alaganan A, Ghosh A, Saito A, van Kuppevelt T, Jacobs-Lorena M. Plasmodium falci-parum ookinetes require mosquito midgut chon-droitin sulfate proteoglycans for cell invasion. Proc Natl Acad Sci USA 2007;104(40):15882-15887.

25. Rosenfeld A, Vanderberg JP. Plasmodium berghei: induction of aminopeptidase in malaria-resistant strain of Anopheles gambiae. Exp Parasitol 1999; 93(2):101-104.

26. Taylor A. Aminopeptidases: structure and function. FASEB J 1993;7(2):290-298.

27. Hooper NM. Families of zinc metalloproteases. FEBS Lett 1994;354(1):1-6.

28. Mohammadzadeh Hajipirloo H, Edrissian GhH, Nateghpour M, Basseri H, Eslami MB. Effects of anti-mosquito salivary glands and deglycosylated midgut antibodies of Anopheles stephensi on fe-cundity and longevity. Iranian J Publ Heal 2005; 34(4):8-14.

29. Dinglasan RR, Kalume DE, Kanzok SM, Ghosh AK, Muratova O, Pandey A, et al. Disruption of Plasmodium falciparum development by antibodies against a conserved mosquito Midgut antigen. Proc Natl Acad Sci U S A 2007;104(33):13461-13466.

30. Klemba M, Gluzman I, Goldberg DE. A Plasmo-dium falciparum dipeptidyl Aminopeptidase I par-ticipates in vacuolar hemoglobin degradation. J Biol Chem 2004;279(41):43000-43007.

31. Taylor A, Carpenter FH, Wlodawer A. Leucine aminopeptidase N (bovine lens): an electron micro-scopic study. J Ultrastruct Res 1979;68(1):92-100.

32. Nishizawa R, Saino T, Takita T, Suda H, Aoyagi T. Synthesis and structure-activity relationships of be-statin analogues, inhibitors of aminopeptidase. J Med Chem 1977;20(4):510-515.

33. Allen MP, Yamada AH, Carpenter FH. Kinetic parameters of metal-substituted leucine aminopep-tidase from bovine lens. Biochemistry 1983;22 (16):3778-3783.

34. Garcia-Alvarez N, Cueva R, Suarez-Rendueles P. Molecular cloning of soluble aminopeptidases from Saccharomyces cerevisiae. Sequence analysis of aminopeptidase ysc II, a putativezinc-metallo pep-tidase. Eur J Biochem 1991;202(3):993-1002.

35. Xu W, Li Q. Progress in the development of amino-peptidase N (APN/CD13) inhibitors. Curr Med Chem Anticancer Agents 2005;5(3):435-476.

36. Ito K, Nakajima Y, Onohara Y, Takeo M, Naka-shima K, Matsubara F, et al. Crystal structure of aminopeptidase N (proteobacteria alanyl aminopep-tidase) from Escherichia coli and conformational change of methionine 260 involved in substrate recognition. J Biol Chem 2006;281(44):33664-33676.