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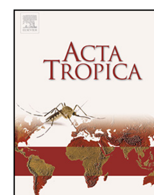

Cloning, characterization and transmission blocking potential of midgut carboxypeptidase A in *Anopheles stephensi*

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ABSTRACT

Transmission-blocking vaccines (TBV) interrupt malaria parasite transmission and hence form an important component for malaria eradication. Mosquito midgut exopeptidases such as aminopeptidase N & carboxypeptidase B have demonstrated TBV potential. In the present study, we cloned and characterized carboxypeptidase A (CPA) from the midgut of an important malarial vector, *Anopheles stephensi*. ClustalW amino acid alignment and *in silico* 3-dimensional structure analysis of CPA predicted the presence of active sites involved in zinc and substrate binding that are conserved among all the known mosquito species. Real-time PCR analysis demonstrated that CPA is predominantly expressed in the midgut throughout the mosquito life cycle and that this gene is significantly elevated in *P. berghei*-infected mosquitoes compared to uninfected blood-fed controls. The high midgut CPA activity correlated with the prominent mRNA levels observed. Peptide-based anti-CPA antibodies were raised that cross-reacted specifically to ∼48 kDa and ∼37 kDa bands, which correspond to zymogen and active forms of CPA. Further, the addition of CPA-directed antibodies to *P. berghei*-containing blood meal significantly reduced the mosquito infection rate in the test group compared to control and blocked the parasite development in the midgut. These results support further development of *A. stephensi* CPA as a candidate TBV.

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1. Introduction

Global efforts such as long-lasting insecticidal bed-nets, indoor residual spraying with insecticides, rapid diagnostic testing and artemisinin-based combination therapy has reduced mortality rates and malaria disease burden. Despite these measures, 214 million malaria cases were recorded in the previous year (WHO, World Malaria Report, 2015). The size and genetic complexity of the malarial parasite make development of malaria vaccine a cumbersome effort.

The life cycle of the malaria parasite is well characterized in the human host whereas its survival strategies and transmission in the mosquito remain a challenging scientific problem. The malaria parasite has to undergo a series of obligatory developmental processes inside the mosquito vector. Male and female gametocytes fertilize within the lumen of the midgut. The resultant ookinete breaches through the peritrophic matrix and laminar surface of the midgut and differentiates at the basal lamina to form an oocyst. Within each oocyst, asexual multiplications produce thousands of sporozoites which are disseminated into the hemocoel. These sporozoites chemotactically invade salivary gland epithelial cells and are delivered to a new vertebrate host during the succeeding blood meal (Greenwood et al., 2008). Ookinete traversal of the midgut is considered a critical process during transmission that involves specific interactions between the parasite and midgut epithelial surface (Vega-Rodríguez et al., 2014).

With respect to the dual-host life cycle and multiple forms of malaria parasite, an effective vaccine is a combination of pre-erythrocytic, asexual, erythrocytic, and sexual stage components. According to the 2013 update to the Malaria Vaccine Technology Roadmap laid down by World Health Organisation, one of the criteria to be considered in development of the malaria vaccine is the reduction in transmission of the parasite. Transmission-blocking vaccine (TBV) interrupts malaria transmission by targeting the sexual or mosquito stages of the parasite antigens. Mosquito midgut invasion by ookinete is a promising target for transmission-blocking approach as parasite numbers

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undergo a severe bottleneck at this stage (Nunes et al., 2014). Recent studies focused on mosquito-derived antigens such as aminopeptidase N (APN) (Mathias et al., 2012) and carboxypeptidase B (CPB) (Lavazec et al., 2007; Raz et al., 2013) of *Anopheles* spp. have documented their role in parasite development.

Carboxypeptidases are zinc-metalloproteases which cleave a single amino acid from the C-terminus of proteins or peptides. These are further classified as A/B (Digestive) and N/E (Regulatory) carboxypeptidases. Digestive carboxypeptidases are divided into A or B classes depending upon their preference for aliphatic or basic residues respectively (Vendrell et al., 2000). In hematophagous insects, the CPA activity is significantly high post blood meal, and the gene sequences encoding CPA have been reported in various mosquito species (Ramos et al., 1993; Edwards et al., 1997; Jahan et al., 1999; Noriega et al., 2002).

Lavazec et al. (2005) characterized carboxypeptidase B (abbreviated as cpbAg1) from the *A. gambiae* midgut and later in 2007 showed that the anti-CPB polyclonal antibodies could inhibit parasite development in the mosquito midgut. Raz et al. (2013) cloned the second CPB (abbreviated as cpbAs1) from *A. stephensi* midgut and demonstrated its transmission blocking potential. In the present study, we characterized CPA and evaluated its competency as a TBV target in *A. stephensi* vector which is predominant in the Indian subcontinent and also spread across the Middle East and South Asia. Notably, CPA has not been evaluated previously for its transmission blocking potential.

2. Materials and methods

2.1. *P. berghei maintenance and infection*

P. berghei ANKA strain was maintained through serial passage in 3–4 week-old female BALB/c mice or as frozen stocks. Mice with parasitemia between 4% and 8% confirmed by Giemsa staining and 2–3 exflagellations/field observed under 100× magnification were used to infect mosquitoes. Female anopheles mosquitoes were reared at 27 °C and 70% humidity with a 12 h light-dark period on 10% sucrose solution under standard laboratory conditions. Mosquitoes were infected with *P. berghei* by feeding on anesthetized infected BALB/c mice for 15 min. Control and infected mosquitoes were placed in a chamber maintained at 21 °C and 70% humidity. *P. berghei* midgut infection was confirmed by monitoring chitinase mRNA expression using quantitative real-time RT-PCR (qRT-PCR). All animal experiments were performed in accordance with the guidelines of the Committee for the Purpose of Control and Supervision on Experiments in Animals (CPCSEA, India) as well as approval of the Institutional Animal Ethics Committee (University of Hyderabad, India).

2.2. *Full length cDNA cloning*

Total RNA was isolated from mosquito midguts (n = 20) using TRIzol® (Sigma-Aldrich, USA). The quality of the RNA was analyzed by denaturing formaldehyde-agarose gel electrophoresis and the quantity was determined using NanoDrop™ spectrophotometer (Thermo Fisher Scientific, USA). Five micrograms of total RNA was reverse transcribed to cDNA using Superscript® III first strand synthesis system (Thermo Fisher Scientific, USA). Primers were designed in the conserved regions of CPA based on the genome sequences of the *A. stephensi* strains reported in the vector base. To obtain the full-length cDNA of CPA, 5′ and 3′ RACE reactions were carried out using RACE kit according to the manufacturer's protocol (Clontech Laboratories, Inc.). 5′ RACE was carried out with gene specific reverse primer (GSP) and universal long and short primers, while 3′ RACE was performed with gene specific forward primer and the long and short primers provided in the kit. All the PCR conditions were programmed as specified in the manufacturer's protocol. The amplified products were cloned into p-GEM-T easy vector (Promega, USA) and sequenced. All the primers used in the study are listed in Supplementary Table 1.

2.3. In silico *analysis*

Specific features of the cloned CPA sequence were analyzed using online software tools. The putative signal peptide was predicted using SignalP 4.0 version. TMpred server was used to predict the transmembrane helix. NetNGlyc software was used for identification of potential N-glycosylation site. Clustal Omega tool was used for multiple sequence alignment. The domain architecture and functional motifs in the protein were predicted using InterScanPro5. The 3-dimensional (3D) structure and the ligand binding sites of the protein were predicted using Phyre2 web server and its component software 3D-Ligand-Site respectively. Phyre2 predicts protein structure by a combination of homology and ab-initio techniques in a multi-step process using several tools. The 3D-Ligand-Site tool performs a structural scan of the query structure against structural library to identify homologous protein structures with bound ligands. These ligands are superimposed onto the query structure to predict a ligand binding residues.

2.4. *Real-time PCR analysis*

Control and infected midguts (n = 10) collected at different time points (0, 8, 14, 20 and 26 h) were dissected in ice-cold TRIzol® and the total RNA isolation was carried out immediately. Total RNA was also isolated from various other tissues (midgut salivary gland, ovary, hemolymph and carcass) and developmental stages (post-hatch, larva, pupa and adult male & female). Standard curve for CPA and ribosomal protein S7 (endogenous control gene) was plotted with serial dilutions of specific primers and cDNA. The list of the primers used is provided in Supplementary Table.1. Gene expression was assessed by Power SYBR Green Master mix (Applied Biosystems, USA) in ABI7500 fast real-time PCR system (Applied Biosystems, USA). A 40-cycle two-step PCR was carried out in triplicates in 10 μl reaction volumes containing the following components: 1 μl of cDNA template, 1 μl of forward and reverse primers each and 5 μl of PCR master mix. Dissociation curve analysis was performed to check for specific amplification. The amplification efficiency was 95%–99% with slope of the curve ranging between −3 to −3.3. Relative quantification results were normalized with the endogenous control. Abundance of CPA mRNA was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). RQ Manager 1.2 (Applied Biosystems, USA) was used to compile data from all plates and compare expression levels.

2.5. *Midgut CPA activity*

To assess midgut CPA activity, midguts (n = 50) were dissected from mosquitoes fed on uninfected and *P. berghei* gametocyte-infected blood at various time points (0, 12, 24 and 48 h). Three independent set of experiments were carried out to determine the CPA activity. Briefly, the isolated midguts were homogenized in a buffer (100 mM NaCl, 50 mM HEPES, 100 μM $ZnCl_2$, pH 7.2) and centrifuged at 10,000g for 20 min at 4 °C. Activities were assayed using 1 mM hippuryl-phenylalanine (Sigma-Aldrich, USA) as the CPA substrate. The reaction was initiated by the addition of 20 μl of midgut extract incubated at 25 °C for 5 min and the absorbance was measured at 254 nm immediately. One unit of enzyme hydrolyzes 1 μmole of hippuryl-L-phenylalanine per minute at pH 7.5 at 25 °C (Bergmeyer et al., 1974).

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1          acatgggggtacagtcgaacgaacggtcgattaaac

38   atggtgtggaacggtggcttctcccggtgtgcggtggccttggtggccgtggtggccttcggtggtgta
     M  V  W  N  G  G  F  S  R  C  A  V  A  L  V  A  V  V  A  F  G  G  V
107  gcgatcgaggcggccgaagtcgcacggtacgataactaccggctgtaccgtgtgacaccccagagcgaa
     A  I  E  A  A  E  V  A  R  Y  D  N  Y  R  L  Y  R  V  T  P  Q  S  E
176  gagcagctgagggcggtcgcagcgatggagcaggcgagcgacagtttaatattcctcgaaacggcccgg
     E  Q  L  R  A  V  A  A  M  E  Q  A  S  D  S  L  I  F  L  E  T  A  R
245  aaggtgggcgatcggttcgacatcgtcgtggcaccgcacaagctcgctgactttacggaaacgctcgaa
     K  V  G  D  R  F  D  I  V  V  A  P  H  K  L  A  D  F  T  E  T  L  E
314  gcggactacatccagcaccaggtgatcgacgagaatgtgcagaatgcgttcgatcaggagcgcacccgg
     A  D  Y  I  Q  H  Q  V  I  D  E  N  V  Q  N  A  F  D  Q  E  R  T  R
383  atcacgaacaagcgtgcaaagggaacgttcgactggagcgactatcacacgctggaggaggtccatgcc
     I  T  N  K  R  A  K  G  T  F  D  W  S  D  Y  H  T  L  E  E  V  H  A
452  tggttggacaagctggcgagtgagcacagtgaggtggaactgttggacgctggtcgatcgcaccagaac
     W  L  D  K  L  A  S  E  H  S  E  V  E  L  L  D  A  G  R  S  H  Q  N
521  cgtacgttgaagggtgtgaagttgtcgtacggtgagggacgaccgggtgtgttcattgagggtggtact
     R  T  L  K  G  V  K  L  S  Y  G  E  G  R  P  G  V  F  I  E  G  G  T
590  catgcgcgagaatggatctctccggacactgtgacgtatatcctgaacgagctggtcaacagtgaagat
     H  A  R  E  W  I  S  P  D  T  V  T  Y  I  L  N  E  L  V  N  S  E  D
659  gcgcaggtccgagcgctagcggaaaagttcgactggtacgtgtttcccagcgtgaacccggacggatat
     A  Q  V  R  A  L  A  E  K  F  D  W  Y  V  F  P  S  V  N  P  D  G  Y
728  gcttacaccttccaggtgaaccgattgtggcgcaagacccgcaaaccttacggtccattctgctatgga
     A  Y  T  F  Q  V  N  R  L  W  R  K  T  R  K  P  Y  G  P  F  C  Y  G
797  gctgatccgaaccggaactgggacttccattgggcggaacagggcaccagcaataatgcttgcgcggat
     A  D  P  N  R  N  W  D  F  H  W  A  E  Q  G  T  S  N  N  A  C  A  D
866  acgtacgctggaccgcaagcattctccgaagtcgagactcgctcgctgtccgcattcgttgaaaagctg
     T  Y  A  G  P  Q  A  F  S  E  V  E  T  R  S  L  S  A  F  V  E  K  L
935  cgtggaaagctcggtgcttacatcgcgttccattcctactctcagctgcttctcttcccgtacggacac
     R  G  K  L  G  A  Y  I  A  F  H  S  Y  S  Q  L  L  L  F  P  Y  G  H
1004 accggagagcactcgccgaatcataaggatctgaacgagatcgcggaagctacggttaaatcgttggcc
     T  G  E  H  S  P  N  H  K  D  L  N  E  I  A  E  A  T  V  K  S  L  A
1073 aaacggtacggcactcagtacaaatatggcaatgtgtacgatgcgatctatccggccagtggttccagc
     K  R  Y  G  T  Q  Y  K  Y  G  N  V  Y  D  A  I  Y  P  A  S  G  S  S
1142 gtggactggagctacggtgcgcaagatgttaagatcgcgtacacgtacgagctgcgtccggattcggat
     V  D  W  S  Y  G  A  Q  D  V  K  I  A  Y  T  Y  E  L  R  P  D  S  D
1211 gcctggaatggatttgtgcttccaccgaatcagatcgtaccaacgggtgaggaaacgttggattcgctc
     A  W  N  G  F  V  L  P  P  N  Q  I  V  P  T  G  E  E  T  L  D  S  L
1280 gttacactgctggaagagtcgtctgcccggggttattacgatgcgaagaattgaatcttttacagcctt
     V  T  L  L  E  E  S  S  A  R  G  Y  Y  D  A  K  N  *
1349 ttagctgttagtagtgaacaaaaaaaaaaaaaaaaaaaaaa 1390
```

Fig. 1. Full-length sequence of CPA cDNA and its predicted amino acid sequence. Nucleotide and amino acid numbers are shown on the left. The arrows depict the signal-peptide cleavage site, and the zymogen activation site in order. The amino acids that are predicted to include the active site and involved in zinc binding are shown in square boxes. Residues involved in substrate binding and cleavage are shown in triangle boxes. The putative N-glycosylation site is represented in circle. The arthropod initiation site, start and stop codons are underlined in order. The lowercase letters at the beginning and the end of the sequences are related to 3′ and5′ untranslated regions, respectively.

2.6. Peptide-based anti-CPA-antibody

Based on the CPA sequence, three different peptides (14 amino acids residues each, >98% purity) were selected using the antigenicity score (information provided in supplementary Table 2) and were conjugated to Keyhole Limpet Hemocyanin (KLH) carrier protein commercially. Each KLH-conjugated antigen was injected into a New Zealand White Rabbit for raising anti-CPA antibody. Prior to immunization, the rabbit were bled for preimmmune sera collection. The ELISA titer for preimmune sera and affinity-purified antibody against the most specific peptide-based antigen is now provided in supplementary Table 3. All the three purified IgG fractions were tested for cross-reactivity against midgut CP-A by immunoblot analysis and the antibody with highest specificity was used for further studies (which is raised against the peptide: RTRITNKRAKGTFDC).

2.7. Immunoblot analysis

Protein homogenate was prepared by homogenization of midgut in 25 mM Tris-HCl (pH 8) and further centrifugation at 20,000*g* at 4 °C for 15 min. The proteins were separated on 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel under reducing conditions and blotted onto a polyvinylidene difluoride membrane (Hybond-P; Amersham). The blotted membrane was saturated with 10% skimmed milk in TBST (tris-buffered saline with 0.1%Tween-20, pH 8.0) at 4 °C for 12 h. The membranes were washed and incubated with the purified IgG fraction (1:2000). Antibody binding was detected by incubating the membrane with goat anti-rabbit alkaline phosphatase secondary antibody (Santa Cruz; dilution, 1:5000) and subsequent treatment with a chromogenic substrate.

2.8. Artificial membrane feeding assay

Membrane feeding assay was performed according to the methods described elsewhere (Tchuinkam et al., 1993; Xi et al., 2007). The CPA antiserum/preimmune serum mixed to either infective/non-infective blood were fed to mosquito groups (**control group** consists of preimmune serum mixed with either infective/non-infective blood and the **test group** consists of CP-A antiserum mixed with either infective/non-infective blood) through a membrane feeder. The antisera used was obtained from single rabbit and tested in three independent experiments. To monitor the effect of anti-CPA antibodies on sexual parasite devel-

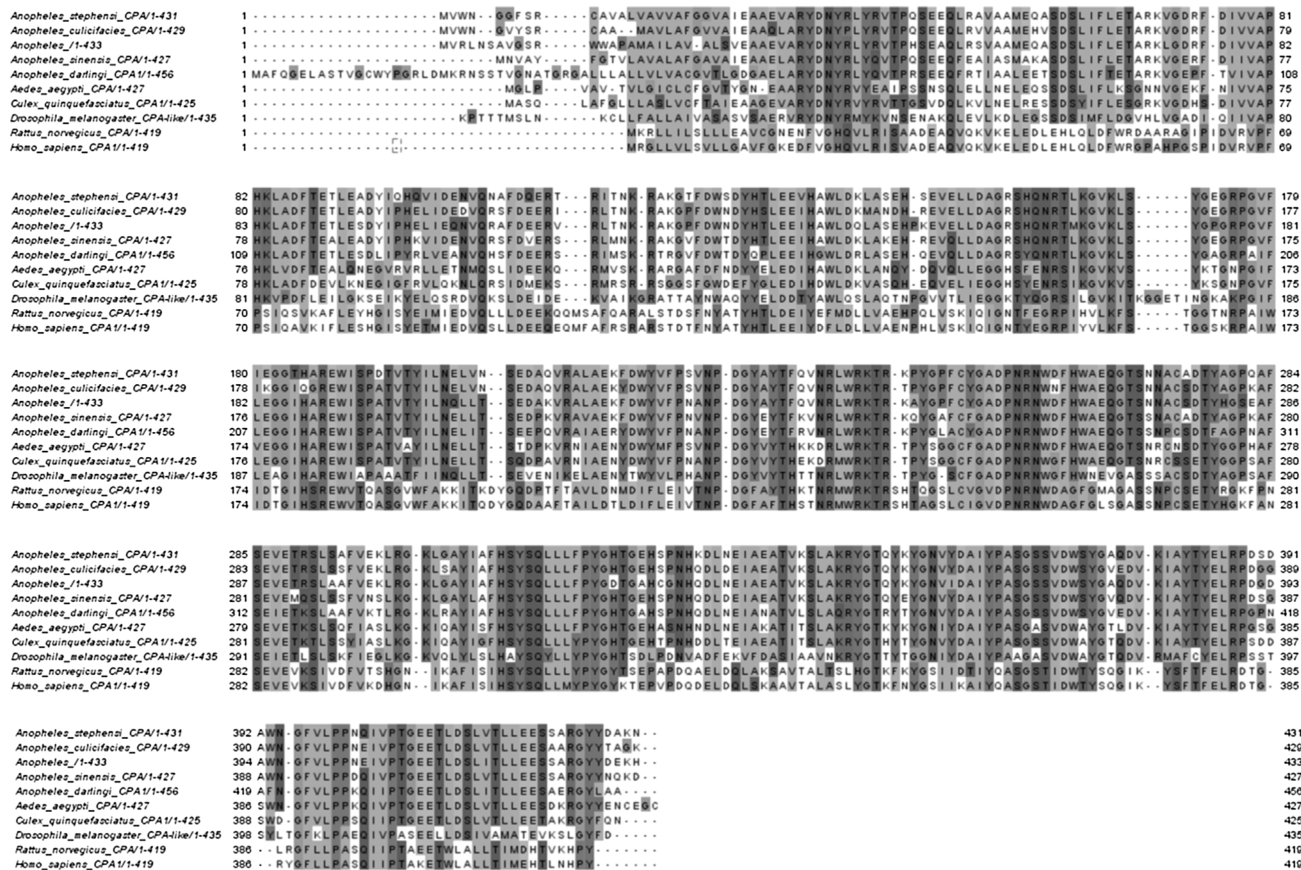

Fig. 2. Alignment of *A. stephensi* CPA with the other known CPA sequences. The residues that are involved in zinc and substrate binding are shown in boxes. TheGenBank nucleotide sequence accession numbers of the aligned sequences are as follows: *Anopheles stephensi* (KT122804); *Anopheles culicifacies* (KF500529); *Anopheles gambiae* (AAB96576); *Anopheles sinensis* (KFB35478); *Anopheles darlingi* (ETN60383); *Aedes aegypti* (AAD47827); *Culex quinquefasciatus* (XP_001861970); *Drosophila melanogaster* (ACI16535); *Rattus norvegicus* (NP058694); *Homo sapiens* (NP001859).

opment, the frequency of infected mosquitoes *i.e.*, total number of uninfected *vs* infected mosquitoes after membrane feeding in control/test and the intensity of infection *i.e.*, the number of oocysts post-blood feeding was monitored.

2.9. Statistical analysis

Data are expressed as mean ± SEM of three independent experiments. Number of samples/repeats for each experiment/assay is indicated in the figure legends. Differences between groups were evaluated using Student's *t*-test or one-way analysis of variance (ANOVA) with Tukey's post-hoc analysis. For each test, *P* values < 0.05 (*) were considered significant.

3. Results

3.1. CPA mRNA and amino acid sequence analysis

A partial fragment of CPA cDNA was cloned from the midgut using degenerate primers and subsequently a full-length cDNA of CPA was obtained using 5′ and 3′ RACE strategy. The nucleotide sequence of CPA was deposited in GenBank (KT122804). The total mRNA length of *A. stephensi* midgut CPA was found to be 1.39 kb with an open reading frame of 1296 bases that encode a polypeptide of 432 amino acids with a theoretical molecular mass ∼48 kDa. However, this protein is translated as a preproprotein with a putative signal sequence from amino acids 1 to 27 and a prodomain from 28 to 110. Therefore, the calculated molecular weight of the active protein would be ∼37 kDa. The 5′ UTR region consists of 37 nucleotide bases, while 3′ UTR region consists of 57 bases. The 5′ UTR region harbors an arthropod initiation sequence. A potential N-glycosylation site is also found towards the C-terminus (Fig. 1). Homology analysis against genomes sequences of the Indian and SDA-500/Pakistan strain reported in the vectorbase and the cloned CP-A sequence (coding sequence) was 99% (at nucleotide level) and 100% (at amino acid level) indicating that there is no variation between these CPA sequences. Further, *A. stephensi* CPA shows substantial amino acid sequence similarity with that of other mosquito species including *A. culicifacies* (88%), *A. gambiae* (86%), *A. sinensis* (85%), *A. darlingi* (76%) and *A. aegypti* (65%). CPA belongs to M14 family in the Barrett-Rawlings-Woessner classification (Barrett et al., 1998) which is the most comprehensively studied group of enzymes among metallocarboxypeptidases. CPA sequence consists of a highly conserved zinc binding motif, HXXE···H, where the three zinc ligands are a histidine, a glutamate harbored two residues downstream and a second histidine located 108–135 amino acids further towards the C-terminus from the first histidine. All the three zinc-binding motifs of CPA are well conserved among all the species. A number of amino acids that are essential for substrate binding and catalysis were found in similar locations of all the aligned CPA sequences (Fig. 2). The 3D structures of *Anopheles* CPAs were generated by Phyre2 web server (Kelly and Sternberg, 2009). In case of *A. stephensi* CPA, 91% residues were modeled with 100% confidence using a structure of human procarboxypeptidase A2 as

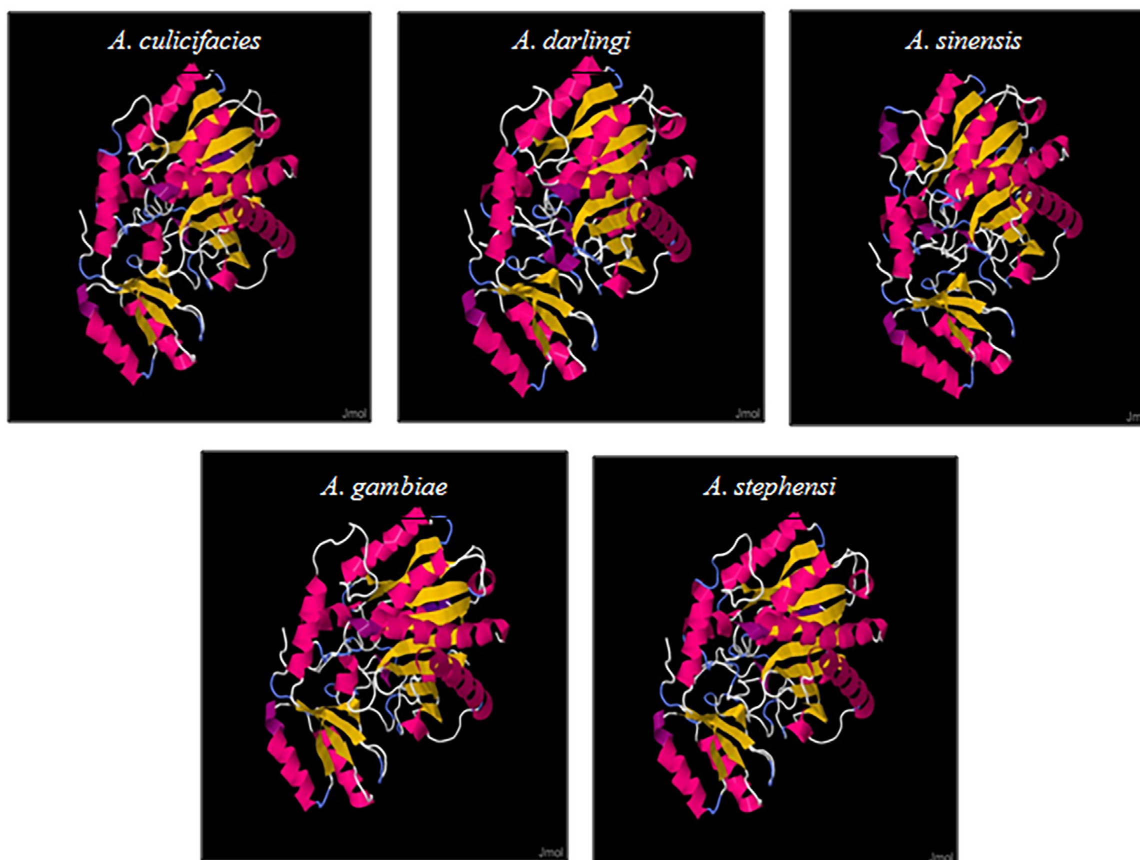

Fig. 3. Predicted 3D structures of CPAs of *A. stephensi*, *A. culicifacies*, *A. gambiae*, *A. sinensis* and *A. darlingi* modeled with human preprocarboxyeptidaseA. Protein structures were predicted using Phyre2 server.

a template. The residues at 185(H), 188(E), 310(H), and 385(E) positions in the modeled structure were predicted to form an active site for zinc binding by 3DLigandSite software. In the resulting 3D structure of the zinc-peptidase complex, the distance between first three residues from the zinc ion was 0 Å whereas 0.61 Å for glutamate at position 385. It suggests that the first three residues in the predicted site are more likely zinc binding, whereas glutamate at position 385 is a potential false positive. It is consistent with the previous findings on zinc binding motifs in CPA peptidases. The topology (arrangement of alpha helices and beta sheets) of remaining CPA structures was very similar (Fig. 3).

3.2. Correlation between mRNA expression and enzyme activity of CPA during infection

The expression pattern of CPA was monitored in the uninfected and infected midguts at different time points using qRT-PCR. The selected time points correspond to the period of transformation of ingested gametocytes from zygotes to ookinetes in the midgut lumen (8–14 h), and high abundance of ookinetes in the midgut lumen (20–26 h). The expression of CPA differed substantially in unifected and infected blood-fed mosquitoes. CPA expression was maximumt at 14 h with approximately a two-fold increase compared to the control. Thereafter, the upregulation of CPA remain elevated until 26 h (Fig. 4A).

To determine if CPA upregulation in *P. berghei*-infected mosquitoes correlates with the enzyme activity, CPA activity was monitored in the infected and uninfected mosquito midguts. As depicted in Fig. 4B, CPA activity in the midguts of mosquitoes fed on *P. berghei* gametocytes was higher than that of the control midguts. At 14 h, CPA activity increased prominently in mosquitoes fed on infected blood. The peak of enzymatic activity at 14 h PBM overlaps the peak of expression observed for CPA in infected mosquitoes. In contrast, maximum CPA activity in uninfected samples was observed at 24 h PBM. Together, these data indicate that ingestion of *P. berghei* gametocytes triggers an upregulation of expression of CPA, and subsequent increase of CPA activity in *A. stephensi* midguts, exerting intense effect at 14 h PBM, a period that corresponds to the transformation of zygotes to ookinetes in the midgut lumen.

3.3. Tissue-specific and developmental expression of CPA

CPA transcript was predominantly expressed in the midgut. The expression was negligible in the other tissues examined (*i.e.* salivary gland, ovary, hemolymph and carcass) (Fig. 5A). Further, the expression of CPA was monitored during *A. stephensi* development and it was found that CPA is constitutively and equally expressed in all the stages of mosquito development *i.e.* post-hatch, larvae, pupae and adult forms (Fig. 5B).

3.4. Immunoblot analysis of midgut CPA using peptide-based antibody

The specificity of the anti-CPA antibodies raised against selected peptides was assessed by western blot analysis. CPA antibody raised against the 14-amino acid peptide 'RTRITNKRAKGTFDC' displayed high specificity for CPA (data not shown) which was used in further investigations. Two bands similar to 48 kDa and 37 kDa were recognized in the mosquito midgut extracts which correspond to the expected size of the zymogen and the active form of CPA (Fig. 6A).

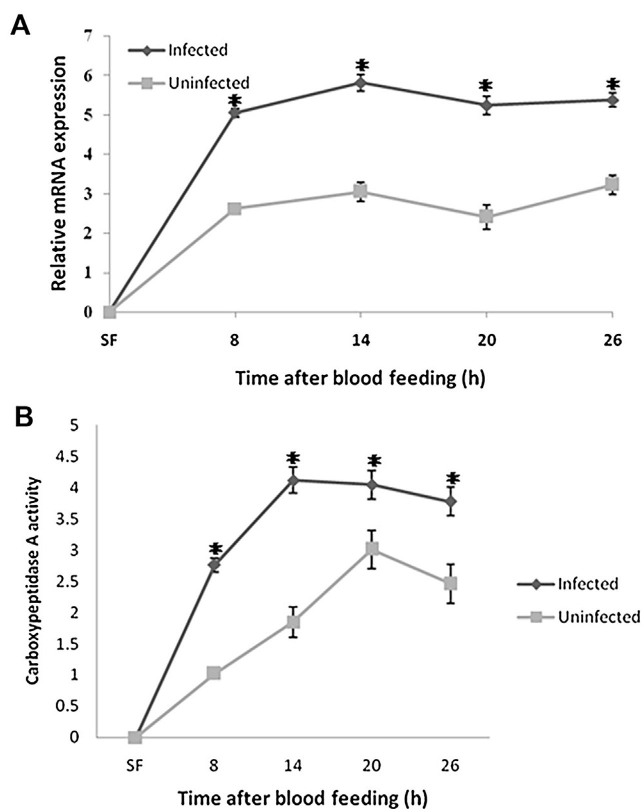

Fig. 4. A) Quantitative analysis of CPAexpression in non-infected *vs P. berghei*-infected midguts at 0, 8, 14, 20 and 26 h B) Carboxypeptidase activity in non-infected *vs P. berghei*-infected midguts at 0, 8, 14 h, 20, and 26 h after ingestion. One unit of enzyme activity is defined as 1 μmol of amino acids released/min. CPB activity in midguts isolated from three independent infection experiments and controls was analyzed. Values are mean ± SEM for n = 3. P value < 0.05 (*) was considered significant.

3.5. CPA-directed antibodies block parasite transmission

To determine the effect of anti-CPA antibodies on *P. berghei* sexual development in the midgut of *A. stephensi*, we used immunized and control (preimmune) rabbit serum. Our results demonstrated that the addition of CPA antiserum to infected blood inhibited the parasite development and reduced the infection rate to 16% in the test group compared to 81% observed in the control group (Fig. 6B). Further, the number of oocysts that emerged out of infected mosquitoes in the test group fed with anti-CPA antibodies were very few in number when compared with the infected mosquitoes in the control group that were fed on the same infected blood (Fig. 6B).

4. Discussion

In the present study, three main findings validate that the cloned full length gene encodes carboxypeptidase A 1) The cloned gene has high similarity to all the reported mosquito carboxypeptidase A sequences including distantly related organisms (35% to *Rattus norvegicus* and 36% to *Homo sapiens*) 2) The cloned gene has conserved functional residues essential for zinc binding and enzymatic activity which are conserved across vertebrate carboxypeptidase A sequences 3) The three-dimensional conformation of all the *Anopheles* CPAs is similar. Significant levels of CPA in male midguts and during larval and pupal development indicate that this molecule has wider functions in the mosquito besides being involved in blood digestion. To the best of our knowledge, this study is the first to characterize CPA and elucidate its role in parasite development in any mosquito species.

During its life cycle, female anopheles mosquito requires digestion of two different food sources, nectar sugars and blood, which necessitates alterations in the types of enzymes present within the midgut. An upregulation of endopeptidases such as trypsin, chymotrypsin as well as exopeptidases such as aminopeptidases and carboxypeptidases is essential to digest and metabolize the protein content present in the blood meal. Hence, three scenarios could be possible during the blood meal digestion i) expression of exopeptidases followed by endopeptidases or ii) expression of endopeptidases followed by exopeptidases iii) expression of both *exo*- and *endo*-peptidases at a time. Early induction of carboxypeptidases including CPA after a blood meal is reported in *A. gambiae*, *A. stephensi* and *A. culicifacies* that trigger the endoproteolytic activity by trypsins and chymotrypsins (Edwards et al., 1997; Jahan et al., 1999; Kumar et al., 2014). In contrast, *A. aegypti* CPA was only expressed at later stages to hydrolyse the products released by the action of early endopeptidases (Edwards et al., 2000). Further, in A. gambiae midgut, the concerted expression of genes for both early *endo*- and *exo*-peptidases was observed (Lavazec et al., 2005). Based on these reports, it can be speculated that the digestive process in the mosquito is regulated by an elaborate biphasic expression pattern of both early and late proteases that reflects a finely tuned adaptation of the mosquito to its hematophagous life.

Evidences suggest that the genome of *Plasmodium* lacks exopeptidases including carboxypeptidases that are otherwise expressed in other stages (hepatic and erythrocytic) outside mosquitoes. Particularly, during erythrocytic stages, these proteases play an important role in hemoglobin catabolism and RBC lysis. Lavazec and Bourgouin (2008) observed that supplementation of the *P. falciparum*-infected blood with basic amino acids, Arginine and Lysine, increased the infection rate and also the efficiency of parasite development in *A. gambiae*. Therefore, it is possible that a portion of the parasite nutritional requirement is supplemented by free amino acids generated by mosquito exopeptidases.

Both expression and activity pattern analysis of CPA monitored at different time points showed that the presence of sexual stages of *P. berghei* triggers its upregulation in the mosquito midgut lumen. Further, as the serum was obtained from the same rabbit (before and after immunization with CPA peptide antigen), it can be assumed that this upregulation is related to the presence of *P. berghei* gametocytes in the mosquito midgut.

To date, research has focused predominantly on parasite-derived molecules such as ookinete surface protein, Pfs25 (Lensen et al., 1992). Few reports documented the viability of a TBV based on mosquito-derived molecules using whole midgut extracts to generate polyclonal antibodies or monoclonal antibodies in various mosquito-*Plasmodium* systems (Ramasamy and Ramasamy, 1990; Lal et al., 1994, 2001). Recently, it was demonstrated that aminopeptidase N from *A. gambiae* (Mathias et al., 2012) and carboxypeptidase B from both *A. gambiae* (Lavazec et al., 2007) and *A. stephensi* (Raz et al., 2013) could be ideal candidates for a TBV. Our data show that CPA fulfills all the essential criteria for a TBV candidate. In addition, CPA is a mosquito-derived antigen, which is not under the selection pressure of the human immune system. The functional residues of CPA are conserved with human CPA; hence, compounds targeted against mosquito CPA's active site could bind to both mosquito and human CPA. However, the human and mosquito CPA shared 36% sequence identity which is sufficient to support differential screening for compounds that could bind specifically to mosquito CPA (Mongkol et al., 2015).

In conclusion, high conservation of the midgut CPA in the mosquitoes including *Anopheles*, *Aedes* and *Culex* species, and the ability of these mosquitoes to transmit multiple species of human malaria parasites, suggests that *A. stephensi* CPA could be a good

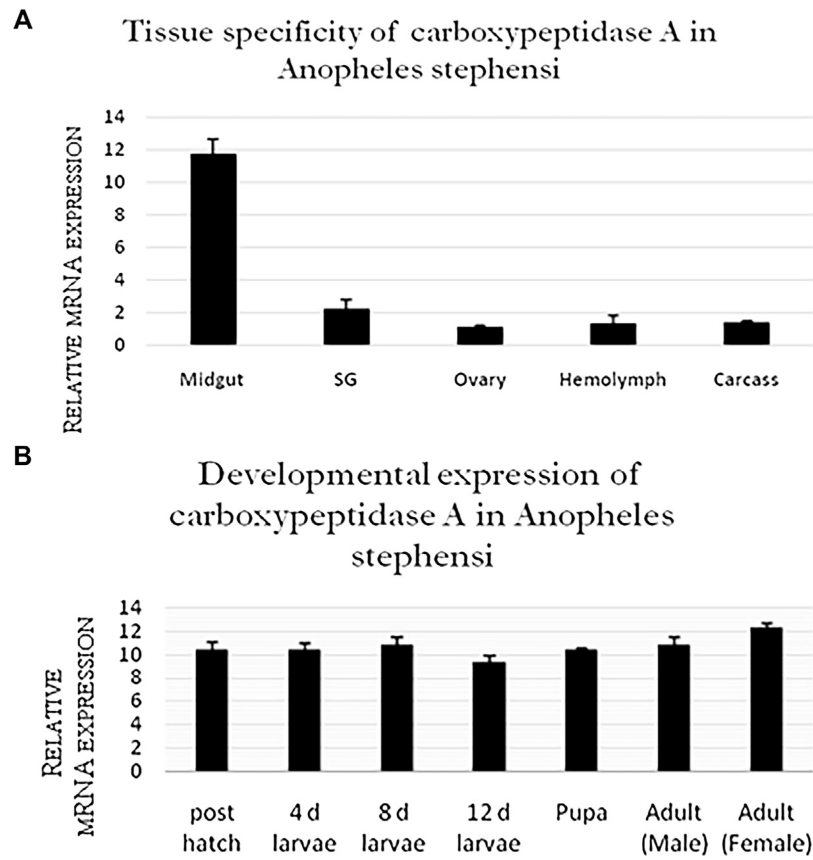

Fig. 5. A) Quantitative expression of *A.stephensi* CPA in various tissues (midgut, salivary gland, ovary, hemolymph and carcass) B) Stage-dependent expression of CPA during *A. stephensi*. development. Each value is expressed as mean ± SEM, n = 3.

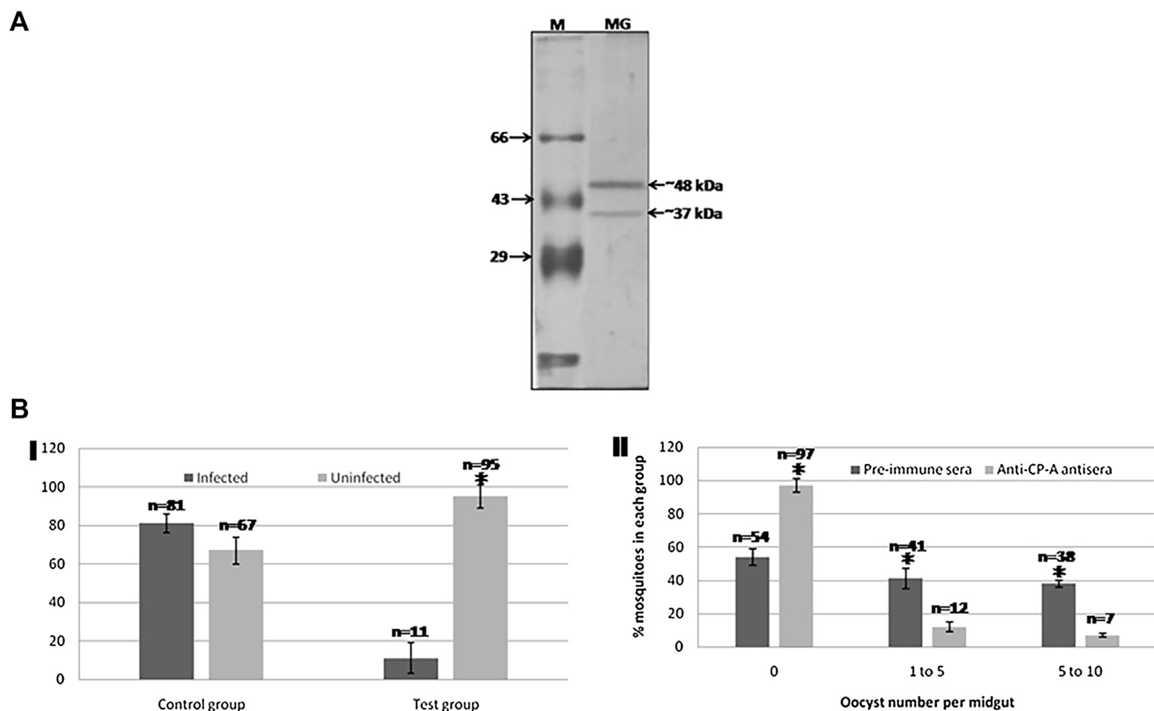

Fig. 6. A) Western analysis of sugar-fed *A. stephensi* midgut using the anti-peptide antibody. Lane 1: Marker; Lane 2: midgut homogenate (40 μg). Note two specific bands corresponding to the zymogen (~48 kDa) and mature (~37-kDa) forms of CPA protein when probed with anti-CPA antibody (depicted by arrows) B) Evaluating the effect of anti-CPA antibodies on sexual *P. berghei* development I) The frequency of infected mosquitoes and II) intensity of infection was evaluated after infection. The proportion of the infected mosquitoes was determined by mean value from each series of three independent infections. Asterisks indicate statistical significance ($P < 0.05$). Numbers above the columns indicate the sample size.

candidate for TBV development in the regions where this vector is an endemic.

Conflict of interest

All the authors declare that there is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.actatropica.2016.12.035.

References

Barrett, A.J., Rawlings, N.D., Woessner, J.F., 1998. Handbook of proteolytic enzymes. Academic Press London.

Bergmeyer, H.U., Gawehn, K., Grassl, M., 1974. In: Bergmeyer, H.U. (Ed.), Methods of Enzymatic Analysis, vol. I, 2nd ed. Academic Press, Inc., New York, NY, pp. 436–437.

Edwards, M.J., Lemos, F.J., Donnelly-Doman, M., Jacobs-Lorena, M., 1997. Rapid induction by a blood meal of a carboxypeptidase gene in the gut of the mosquito *Anopheles gambiae*. Insect Biochem. Mol. Biol. 27, 1063–1072.

Edwards, M.J., Moskalyk, L.A., Donelly-Doman, M., Vlaskova, M., Noriega, F.G., Walker, V.K., Jacobs-Lorena, M., 2000. Characterization of a carboxypeptidase A gene from the mosquito *Aedes aegypti*. Insect Mol. Biol. 9, 33–38.

Greenwood, B.M., Fidock, D.A., Kyle, D.E., Kappe, S.H., Alonso, P.L., Collins, F.H., Duffy, P.E., 2008. Malaria: progress, perils, and prospects for eradication. J. Clin. Invest. 118, 1266–1276.

Jahan, N., Docherty, P.T., Billingsley, P.F., Hurd, H., 1999. Blood digestion in the mosquito, *Anopheles stephensi*: the effects of *Plasmodium yoelii nigeriensis* on midgut enzyme activities. Parasitology 119, 535–541.

Kumar, A., Sharma, A., Sharma, R., Gakhar, S.K., 2014. Identification, characterization and analysis of expression of gene encoding carboxypeptidase A in *Anopheles culicifacies* A (Diptera: culicidae). Acta Trop. 139, 123–130.

Lal, A.A., Schriefer, M.E., Sacci, J.B., Goldman, I.F., Louiswileman, V., Collins, W.E., Azad, A.F., 1994. Inhibition of malaria parasite development in mosquitoes by anti-mosquito-midgut antibodies. Infect. Immun. 62, 316–318.

Lal, A.A., Patterson, P.S., Sacci, J.B., Vaughan, J.A., Paul, C., Collins, W.E., Wirtz, R.A., Azad, A.F., 2001. Anti-mosquito midgut antibodies block development of *Plasmodium falciparum* and *Plasmodium vivax* in multiple species of *Anopheles* mosquitoes and reduce vector fecundity and survivorship. Proc. Natl. Acad. Sci. U. S. A. 98, 5228–5233.

Lavazec, C., Bourgouin, C., 2008. Mosquito-based transmission blocking vaccines for interrupting *Plasmodium* development. Microbes Infect. 10, 845–849.

Lavazec, C., Bonnet, S., Thiery, I., Boisson, B., Bourgouin, C., 2005. cpbAg1 encodes an active carboxypeptidase B expressed in the midgut of *Anopheles gambiae*. Insect Mol. Biol. 14, 163–174.

Lavazec, C., Boudin, C., Lacroix, R., Bonnet, S., Diop, A., Thiberge, S., Boisson, B., Tahar, R., Bourgouin, C., 2007. Carboxypeptidases B of *Anopheles gambiae* as targets for a *Plasmodium falciparum* transmission-blocking vaccine. Infect. Immun. 75, 1635–1642.

Lensen, A.H., Van Gemert, G.J., Bolmer, M.G., Meis, J.F., Kaslow, D., Meuwissen, J.H., Ponnudurai, T., 1992. Transmission blocking antibody of the *Plasmodium falciparum* zygote/ookinete surface protein Pfs25 also influences sporozoite development. Parasite Immunol. 14, 471–479.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25, 402–408.

Mathias, D.K., Plieskatt, J.L., Armistead, J.S., Bethony, J.M., Abdul-Majid, K.B., McMillan, A., Angov, E., Aryee, M.J., Zhan, B., Gillespie, P., Keegan, B., Jariwala, A.R., Rezende, W., Bottazzi, M.E., Scorpio, D.G., Hotez, P.J., Dinglasan, R.R., 2012. Expression, immunogenicity, histopathology, and potency of a mosquito-based malaria transmission-blocking recombinant vaccine. Infect. Immun. 80, 1606–1614.

Mongkol, W., Arunyawat, U., Surat, W., Kubera, A., 2015. Active compounds against *Anopheles minimus* carboxypeptidase B for malaria transmission-blocking strategy. J. Med. Entomol. 52, 1322–1332.

Noriega, F.G., Edgar, K.A., Bechet, R., Wells, M.A., 2002. Midgut exopeptidase activities in *Aedes aegypti* are induced by blood feeding. J. Insect Physiol. 48, 205–212.

Nunes, J.K., Woods, C., Carter, T., Raphael, T., Morin, M.J., Diallo, D., Leboulleux, D., Jain, S., Loucq, C., Kaslow, D.C., Birkett, A.J., 2014. Development of a transmission-blocking malaria vaccine: progress, challenges, and the path forward. Vaccine 32, 5531–5539.

Ramasamy, M.S., Ramasamy, R., 1990. Effect of anti-mosquito antibodies on the infectivity of the rodent malaria parasite *Plasmodium berghei* to *Anopheles farauti*. Med. Vet. Entomol. 4, 161–166.

Ramos, A., Mahowald, A., Jacobs-Lorena, M., 1993. Gut-specific genes from the black fly *Simulium vittatum* encoding trypsin-like and carboxypeptidase- like proteins. Insect Mol. Biol. 1, 149–163.

Raz, A., Dinparast Djadid, N., Zakeri, S., 2013. Molecular characterization of the carboxypeptidase B1 of *Anopheles stephensi* and its evaluation as a target for transmission-blocking vaccines. Infect. Immun. 81, 2206–2216.

Tchuinkam, T., Mulder, B., Dechering, K., Stoffels, H., Verhave, J.P., Cot, M., Carnevale, P., Meuwissen, J.H., Robert, V., 1993. Experimental infections of *Anopheles gambiae* with *Plasmodium falciparum* of naturally infected gametocyte carriers in Cameroon: factors influencing the infectivity to mosquitoes. Trop. Med. Parasitol. 44, 271–276.

Vega-Rodríguez, J., Ghosh, A.K., Kanzok, S.M., Dinglasan, R.R., Wang, S., Bongio, N.J., Kalume, D.E., Miura, K., Long, C.A., Pandey, A., Jacobs-Lorena, M., 2014. Multiple pathways for *Plasmodium* ookinete invasion of the mosquito midgut. Proc. Natl. Acad. Sci. U. S. A. 111, 492–500.

Vendrell, J., Querol, E., Avilés, F.X., 2000. Metallocarboxypeptidases and their protein inhibitors. Structure: function and biomedical properties. Biochim. Biophys. Acta 1477, 284–298.

Xi, Z., Das, S., Garver, L., Dimopoulos, G., 2007. Protocol for Plasmodium falciparum infections in mosquitoes and infection phenotype determination. J. Vis. Exp. 222, http://dx.doi.org/10.3791/222.