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Short communication

Gene disruption reveals a dispensable role for Plasmepsin VII in the *Plasmodium berghei* life cycle

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ABSTRACT

Plasmepsins (PM), aspartic proteases of *Plasmodium*, comprises a family of ten proteins that perform critical functions in *Plasmodium* life cycle. Except VII and VIII, functions of the remaining plasmepsin members have been well characterized. Here, we have generated a mutant parasite lacking PM VII in *Plasmodium berghei* using reverse genetics approach. Systematic comparison of growth kinetics and infection in both mosquito and vertebrate host revealed that PM VII depleted mutants exhibited no defects in development and progressed normally throughout the parasite life cycle. These studies suggest a dispensable role for PM VII in *Plasmodium berghei* life cycle.

Plasmepsins are aspartic proteases of *Plasmodium falciparum* (*P. falciparum*) that have been extensively studied in blood stages for their role in hemoglobin (Hb) degradation and hence as potential drug targets. *P. falciparum* encodes for ten plasmepsins [1], out of which four paralogues *viz.*, PM I, II, III (HAP) and IV have been shown to reside in the acidic food vacuole of *P. falciparum* infected red blood cells. These plasmepsins orchestrate an ordered process of Hb degradation where PM I and PM II likely catalyze the initial cleavage. Further catabolism of Hb to free amino acids is facilitated by combined action of histoaspartic protease (HAP, PM III), PM IV, cysteine proteases and metalloproteases [2]. Though PM V, IX and X are expressed in the blood stages, they do not have a function in the food vacuole [3]. PM V is a parasite endoplasmic reticulum resident protease [4] that cleaves the export cargo containing PEXEL motif to facilitate their translocation into cytosol to promote virulence and erythrocyte take over [5,6]. Both PM IX and X were shown to localize in trophozoite stage [3]. While PM IX locus is recalcitrant to gene disruption reiterating its essential role in blood stages [7], the function of PM X is not known.

PM VI, VII and VIII are not expressed in the blood stages [3,8] implying a possible role in other stages. An evidence corroborating for an extra erythrocytic function of PM VI was recently reported in mosquitoes stages of *Plasmodium berghei* (*P. berghei*), where depletion of *pm vi* led to absence of salivary gland sporozoites, though functional oocyst were observed [7]. While gene expression data for PM VII in mosquito transmission stages have been reported earlier [9,10] its functional role has not been investigated. A better understanding of its role in other *Plasmodium* stages may provide novel insights into their biological roles unique to these stages.

Towards this end, we have undertaken a genetic approach to investigate the role of *P. berghei* PM VII (PBANKA_051760) in the parasite life cycle. We first analyzed the gene expression of PM VII both in the mosquito and liver stages by quantitative real time PCR. The cDNA samples were prepared at different time points from both mosquito and liver stages as described in supplementary material. Normalized data obtained as a ratio of *P. berghei PM VII*/*P. berghei 18S rRNA* revealed highest level of transcript abundance on day 4 (MSD4) post blood meal. While other time points of mosquito stages showed modest expression, no expression was detected in the liver stages (Fig. 1A). In order to reveal the function of the PM VII, we have generated a loss of function mutant using gene replacement strategy (Fig. 1B). To achieve this, we amplified 650 bp of 5′ and 550 bp of 3′ sequence flanking the target (PBANKA_051760) by PCR. The primer pair CTCGAGGGAGCAATTATGTTACTATATC and ATCGATGGTTTATACACTTGTACGACA were used to amplify the 5′ end and the primer pair GCGGCCGCCCTGAATGGAAAAGAATACATA and GGCGCGCCCCACTATTTAACCACACGATT were used to amplify the 3′ end. The restriction sites in the sequence are underlined.

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0166-6851/

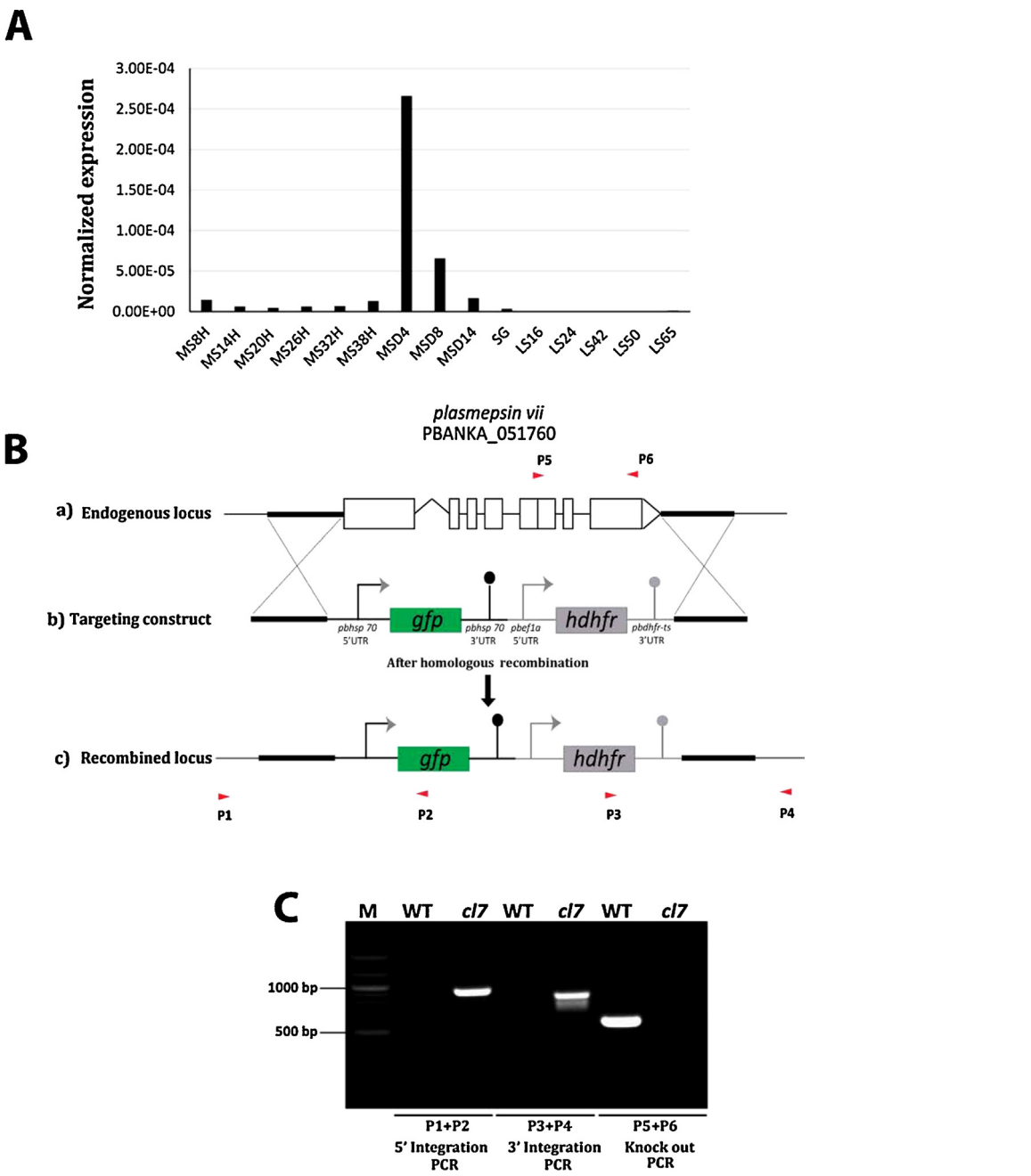

Fig. 1. (A) Quantitative determination of the transcript levels of PM VII in mosquito and liver stages of *P. berghei*. Expression levels were measured in mosquito stages (MS) post infection at hours (H) 8, 14, 20, 26, 32, 38 and on day (MSD) 4, 8, 14, salivary gland sporozoites (SG), and liver stages (LS) at hours 16, 24, 42, 50, 65. Normalization of target gene expression was performed by obtaining a ratio of absolute number of *pm vii*/*Pb 18S rRNA*. (B) Genomic organization of *P. berghei pm vii* and strategy used to replace the target by double cross over recombination (DCO). (a) *P. berghei pm vii* locus. (b) Targeting construct having 650 pb of 5′ and 550 bp of 3′ part of *P. berghei pm vii* cloned on either side of GFP and hDHFR cassette. (c) Recombined locus after DCO. (C) Diagnostic PCR to confirm the site specific integration: Genomic DNA obtained from either WT or *pm vii* KO were subjected to PCR using indicated sets of primers. A PCR product corresponding to 850 bp using P1 + P2 and 750 bp using P3 + P4 in *pm vii* KO confirmed respectively the 5′ and 3′ end specific integration. A wild type specific PCR product corresponding to 550 bp using P5 + P6 confirmed the absence of *pm vii* in the KO.

Following sequence confirmation, the 5′ and 3′ fragments were cloned into pBC-GFP-DHFR vector using restriction sites XhoI/ClaI and NotI/AscI respectively. The targeting vector was linearized using restriction sites XhoI and AscI. Ten micro gram of targeting construct was electroporated into wild type (WT) synchronized blood stage schizonts as described earlier [11] and the transfected parasites were immediately injected intravenously into mouse followed by selection on pyrimethamine. Successful integration of the targeting construct by double cross over recombination was confirmed by diagnostic PCR (Fig. 1C) and also by GFP expression, observed under fluorescent microscope (Nikon Eclipse NiE AR). The *pm vii* KOs derived from two independent transfections were subjected to limiting dilution and two clones were selected for further phenotypic characterization. While identical results were obtained in all experiments using two independent clones (data not shown), we report the results of one clone (cl7) in this study.

Successful generation of loss-of-function mutant indicated that PM VII was dispensable for blood stages. However to determine whether absence of PM VII has any effect on growth of blood stages, 10^3 infected RBC of either WT or *pm vii* KO were intravenously

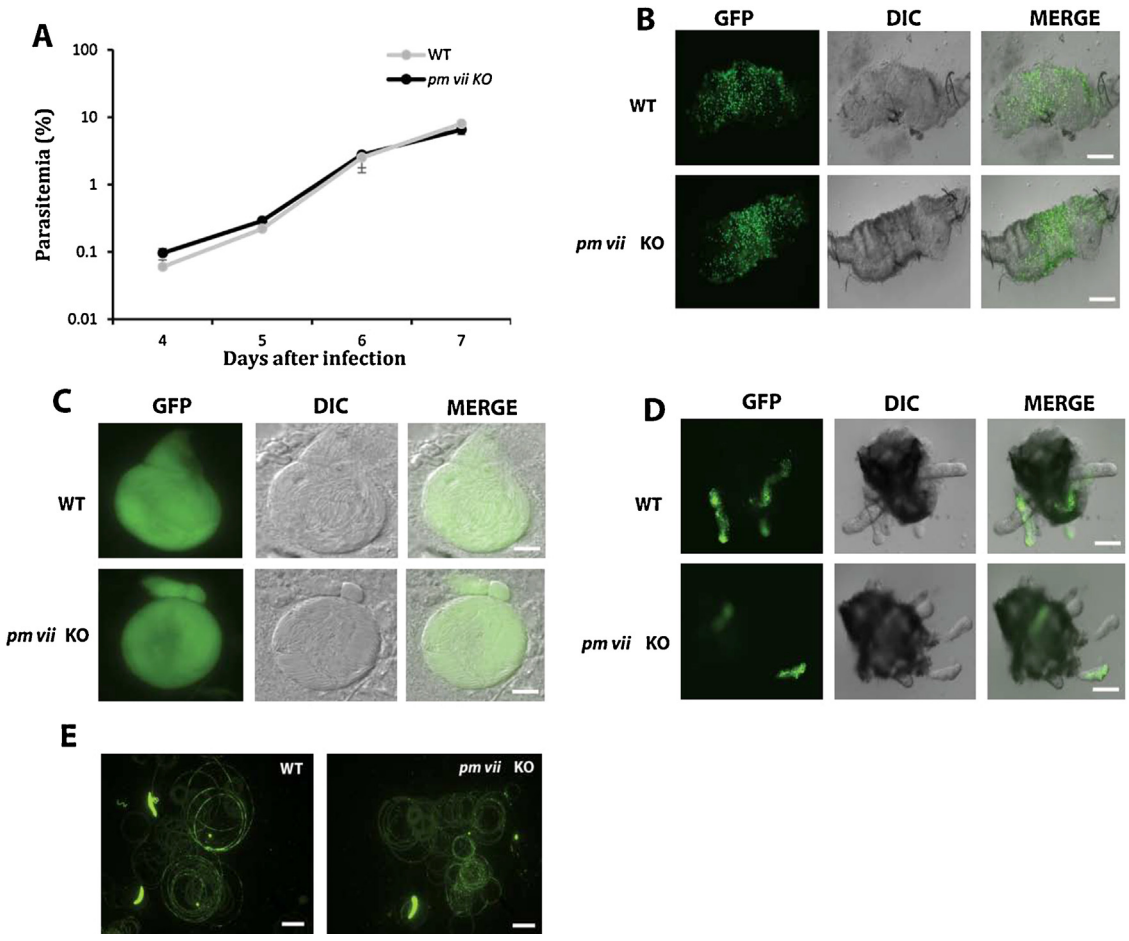

Fig. 2. Phenotypic characterization of *pm vii* KO parasites. (A) Genetic ablation of pm vii does not affect blood stage development. *In vivo* growth curves of wild type (gray) and KO (black) parasites. Female BALB/c mice ($n = 3$) were intravenously injected with 1000 mixed blood stage parasites and parasitaemia was determined by microscopic examination of Giemsa-stained blood smears. Shown values are mean ± standard deviation. (B) Dissected midguts. (C) Magnified oocyst showing sporulation. (D) Dissected salivary glands. (E) Trails released by WT and *pm vii* KO sporozoites during gliding motility on glass slides (scale bars, 200 μm for midguts, 20 μm for oocyst, 200 μm for salivary glands and 10 μm for sporozoites showing gliding motility).

injected into a group of 3 female BALB/c mice and parasitaemia was daily monitored by microscopic examination of Giemsa stained blood smears for a period of 7 days. Both WT and *pm vii* KO blood stages showed similar multiplication rates (Fig. 2A). Further to investigate the role of PM VII in the mosquito stages, we propagated cloned *pm vii* KO parasites into 4–5 female BALB/c mice. GFP expressing WT *P. berghei ANKA* parasites were used as control. All gametocyte positive mice were selected for feeding female *Anopheles stephensi* mosquitoes. The infected mosquitoes were maintained at 20 °C and 75–80% relative humidity (RH). On D14 post blood meal, the mosquito midguts were dissected and the oocyst numbers were determined under fluorescent microscope (Fig. 2B and Table 1A). No apparent differences in morphology were observed in *pm vii* KO oocysts as compared to WT (Fig. 2C). Dissected salivary gland of *pm vii* KO infected mosquitoes contained viable sporozoites whose numbers were comparable to that of the WT sporozoites (Fig. 2D and Table 1A). These results suggest that lack of PM VII did not compromise development of oocysts or ability of haemocoel sporozoites to invade salivary glands.

Table 1A
Oocyst and salivary gland sporozoite numbers was determined at D12 and D21 respectively, post infectious blood meal. Three independent feeding experiments were performed.

Parasites	Number of oocyst/mosquito Mean (±SD)	Number of salivary gland sporozoites/mosquito Mean (±SD)
WT	147 (±70)	14,500 (±3700)
pm vii KO	180 (±80)	16,000 (±3900)

To analyze the characteristics of pre-erythrocytic stages, we studied sporozoite gliding motility by allowing both *pm vii* KO and WT parasites to glide on glass slides coated with 3D11 mAb (anti-PbCS). To visualize the trails of circumsporozoite protein (CSP) released by sporozoites, the slides were fixed with 4% paraformaldehyde, probed with biotinylated 3D11 antibody and revealed by FITC-streptavidin [12]. No differences were observed in the numbers and pattern of trails produced by *pm vii* KO sporozoites as compared to WT (Fig. 2E). To determine if the *pm vii* KO sporozoites successfully initiate blood stage infection *in vivo*, female C57BL/6 mice were infected through mosquito bite. Two groups of three mice were kept on individual cages, each containing 11–16 infected mosquitoes of either WT or *pm vii* KO (D21 post blood meal) to initiate infection. The number of mosquitoes that took blood meal ranged from 54% to 90% in both groups. The number of blood fed mosquitoes that were positive for salivary gland infection ranged from 58% to 100% (Supplementary Table 1), suggesting a successful infection in mice through bite. Analysis of the prepatent period in *pm vii* KO revealed the appearance of blood stage infection on D4 that was similar to wild type parasites (Table 1B). From these studies, we conclude that depletion of PM VII neither alters *in vivo* cell traversal activity or hepatocyte tropism and these sporozoites behave identical to the WT sporozoites with

Table 1B
Monitoring of pre-patent period following mosquito bite. Pre-patent period: after sporozoite inoculation, number of days until the detection of blood stage parasites by microscopy.

Wild type		*pm vii* KO	
Infected/total	Pre-patent period	Infected/total	Pre-patent period
3/3	Day 4	3/3	Day 4

respect to commitment to hepatocyte infection and completion of exo-erythrocytic development.

The role of extra erythrocytically expressed plasmepsins has only started to be explored recently. The first study in this direction was the ablation of PM VI in *P. berghei* that revealed its role in mosquito stages where oocyst developed normally but no salivary gland sporozoites were detected [7]. Based on the presence of transmembrane domain and signal peptide that may facilitate its attachment to plasma membrane, it is predicted that PM VI may be a likely substrate for the romboid-3, a member of intra membrane serine protease family [13]. Such speculation is based on the finding that mutants lacking ROM-3 also manifest identical phenotype as *pm vi* KO where oocyst were produced but no functional sporozoites were observed. While such hypothesis needs validation, these findings suggest a possibility that both PM VI and ROM-3 may play a role in pathway that regulates sporogony in oocyst. Based on the evidence of gene expression data that PM VII is expressed in the transmission stages [9,10] we took a reverse genetics approach to dissect the role of PM VII by generating a KO. By systematic comparison of developmental kinetics of *pm vii* KO and WT parasites in mosquito and vertebrate host, we report that ablation of PM VII had no apparent fitness loss with respect to progression through the life cycle. Our preliminary results indicate a similar pattern of gene expression for PM VII and VIII in the mosquito stages (Babu S. Mastan and Kumar KA, unpublished data). While our studies reveal a dispensable role of PM VII in *Plasmodium* life cycle, further investigation is required to prove if any functional redundancy exists among mosquito stage expressed plasmepsins that may compensate for loss of function.

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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.molbiopara.2014.05.004.

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