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Contributed Paper

Investigation of *Plasmodium vivax* Rhomboid-like Protease 1 Compared to *Plasmodium falciparum* Rhomboid Protease 1 in Erythrocytic Cycle

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ABSTRACT

Plasmodium Rhomboids (ROM) are members of a family of serine proteases that play key roles in erythrocyte invasion. To date, the *P. vivax* ROM-like 1 (PvROM-like 1) protein has not been characterized. This study aimed to detect the rhomboid proteins in the erythrocytic cycle of the malaria parasite. Infected blood samples were collected from malaria clinics in Mae Sot, Tak, Thailand during September 2013 - May 2014. Extracted RNA was examined for the presence of *Pvrom*-like 1 by RT-PCR. The corresponding protein was characterized using SDS-PAGE, Western blot and immunofluorescence assays. The apparent molecular weight of PvROM-like 1 protein was estimated to be approximately 30 kDa using polyacrylamide gel electrophoresis and Western blotting. PvROM-like 1 and PfROM1 proteins could be detected by use of anti-PfROM1 and anti-PbROM1 antibodies, but not with an anti-DroROM1 antibody. Our results show that PvROM-like 1 protein is found in all erythrocytic stages of the *P. vivax* cycle and may function in the invasion of malaria into red blood cells.

Keywords: *Plasmodium vivax* ROM1-like, erythrocytic cycle, Mae Sot, Tak, Thailand

1. INTRODUCTION

Malaria remains a critical public health problem, especially along the Thai border regions. *Plasmodium vivax* is a major malaria species and causes significant human morbidity [1]. The increasing trend of *P. vivax* malaria and the emergence of drug-resistant strains

of the *P. vivax* parasite are major concerns for malaria control. Efforts are being made to develop an effective malaria vaccine and anti-malarial drugs. Information on proteins of *P. vivax* from field populations is essential for these tasks [2]. Characterization of the majority of parasite proteins has been conducted with *P. falciparum* while studies on *P. vivax* are lacking. Because *P. vivax* forms sexual stages transmissible by the mosquito soon after human infection and develops a dormant hypnozoite stage form in the liver, *P. vivax* have become more prevalent. Elimination or reduction of *P. falciparum* might possibly lead to an increase in the number of *P. vivax* cases in the same populations [3]. Some of the protein such as Apical membrane antigen-1 (*AMA1*), Circumsporozoite *protein* (*CSP*), Duffy binding protein (*DBP*) and a new protein, Rhomboid protein (ROM) could affect the severity of the disease as found in an important organelle such as apicomplexan secretory organelles.

Rhomboids are a relatively recently-discovered class of serine proteases that have the unusual property of cleaving its substrates within their transmembrane domains. Firstly discovered in *Drosophila* [4], there is growing evidence that the rhomboids mediate key proteolytic processing activities during the invasion by apicomplexan parasites [5-6]. Apicomplexa parasites carry a set of secretory organelles (rhoptries, micronemes, and dense granules) found at the apical end of these parasites, the end that invades cells of the vertebrate host [7]. Apicomplexa parasites, including *Plasmodium* are obligate intracellular pathogens that must invade host cells to survive [8]. These organelles were first identified by their distinct morphological appearance in transmission electron micrographs [9]. Many parasite proteins required for invasion of erythrocytes are segregated into the micronemes. Rhomboid proteins in Apicomplexa parasites have been studied for the first time in *Toxoplasma gondii*. The TgROM1 was localized mostly to the microneme [10], while TgROM2 was localized mostly to the Golgi [11]. The TgROM4 was localized mostly to the surface and TgROM5 was localized mostly to the posterior end of intracellular parasites [11-12].

Plasmodium ROM1 protein of *P. berghei* [13], *P. yoelii* [14] and *P. falciparum* [15-16] were found to be important in the erythrocyte invasion. However, *P. vivax* ROM-like 1 protein has not been characterized. The purpose of this study was to detect the rhomboid proteins in the erythrocytic cycle of the *P. vivax* compared to *P. falciparum* in erythrocytic cycle.

2. MATERIALS AND METHODS

2.1 Collected Blood Samples

The data were collected by a Cross-sectional study from September 2013 - May 2014. The authors collected 30 clinical suspected malaria blood samples from malaria clinics in Mae Sot, Tak, Thailand. Blood samples were obtained by finger prick and 5 mL of venous blood. Prior to enrollment, all patients signed consent forms which were approved by the Ethics Committee of Ministry of Public Health, Thailand (permission no. 15/2556).

2.2 Diagnosis of Malaria

All malaria patients were symptomatic and the malaria species had been identified by microscopy by staining thin and thick blood smears with Giemsa stain and using molecular diagnosis by performing multiplex PCR [17]. Mixed species infections and asexual parasitemia < 1/200 RBC were not included in the present study.

2.3 RNA Extraction and *Pvrom*-like 1 Amplification

RNA was extracted from asexual blood stage parasites, using TRIzol (Thermo Fisher Scientific, USA) as described earlier [18]. Primers used for *Pvrom*-like 1 were *Pvrom*-like 1 F (5′-CCCCACAAGTGTGCCAATAAA TTCATCCAA-3′) and *Pvrom1*-like 1 R (5′-TCAGCAGGTGCGCGGACGGCGAA CAAAACA3′) which flank exon 1 to exon 4 (GenBank acc. no. XM_001615211). RT-PCR was performed in a 50-µL mixture containing 5× Qiagen OneStep RT-PCR Buffer, 0.2 µM of each primer, 200 µM of each of the four dNTPs, 3 µL of RNA template, 2 µL of RNase inhibitor and 2 µL of Qiagen OneStep RT-PCR Enzyme Mix. Thermal cycling conditions were as follows: reverse transcription at 50 °C for 30 min, initial PCR activation 95 °C for 15 min, followed by 30 cycles of 68 °C for 1 min, 72 °C for 1 min, 94 °C for 1 min followed by a final heating step of 72 °C for 10 min. The PCR products were separated by 1.5% agarose gel-electrophoresis and stained with Gel red (Biotium, USA). The PCR product was then gel-purified using QIA quick gel extraction kit (Qiagen, Germany). DNA (cDNA) sequencing was performed with ABI's 3500XL Genetic Analyzer *(Ramathibodi* Hospital, Thailand).

2.4 Molecular Cloning and Protein Expression of PvROM-like 1

RT- PCR product of *Pvrom*-like 1 was cleaved with *Eco*RI and *Sph*I and inserted into the pGEM-T Easy plasmid (Promega, USA). The pGEM-*Pvrom*-like 1 was transformed into BL21 (DE3) *E. coli* cells (Life BioDynamics Laboratory, Inc.) and the transformants were selected on LB agar plate containing ampicillin (100 µg/ml) and kanamycin (25 µg/ml).

A single colony of *E.coli* harboring pGEM-*Pvrom*-like 1 plasmid was inoculated into 50 ml of LB medium containing ampicillin (100 µg/ml) and kanamycin (25 µg/ml), and grown at 37°C until the OD_{600} reached about 0.5. An aliquot (5ml) of this pre-culture was transferred into 1 liter LB medium containing the same antibiotics, incubated on a shaker at 37°C for 4 h. The recombinant PvROM1-like protein expression was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM when OD_{600} reached 0.5. After 10-12 h of incubation, the bacterial cells were harvested by centrifugation at 5000 g at 4 °C for 45 min. The protein was purified using Ni-NTA Fast start kit (Qiagen, Germany). Protein concentration was determined by the method of Bradford [19] using bovine serum albumin (BSA) as a standard. Lysates of induced and non-induced cells were analyzed on SDS-PAGE [20].

2.5 Antibodies

The following set of antibodies was used in this study. Primary antibodies: Rabbit anti-*Pf*ROM1 peptide pAb was purchased from Genescript (NJ, USA). (The amino acid sequence of the peptide to be used as immunogen QSQSSFVQRSKPIDC and Host Strain: New Zealand Rabbit), Rabbit anti-*Pb*ROM1mAb was supported from Malaria Research Institute, Johns Hopkins Bloomberg School of Public Health (MD, USA) [13]. In brief, antibodies were raised in rabbit against the N-terminal 52 amino acids of PbROM1 expressed in bacteria as a fusion protein using the pBAD expression system (Invitrogen) and Rabbit anti-*Drosophila melanogaster* ROM1 pAb was purchased from Santa Cruz Biotechnology (TX, USA). Secondary antibodies: Alexa Fluor 488 (green), 594 (red) and 350 (blue) conjugated anti-rabbit IgG were purchased from Thermo Fisher Scientific (MA, USA).

2.6 Western bolt Analysis

Purified PvROM-like 1 proteins were separated by SDS-polyacrylamide gel and subjected to western blot analysis as described [21]. Briefly, proteins were transferred from gel on to a PVDF membrane and were blocked with 5% Skim milk in 1x PBS. The blot was then incubated with Rabbit anti-PfROM1 peptide pAbs from Genescript (NJ, USA) diluted 1:1,000 as a primary antibody targeting PvROM-like 1 followed by incubation with anti-rabbit linked HRP (New England Biolabs, UK). The blot was developed by using the West-Pico kit (Thermo Fisher Scientific, USA) as per manufacturer's instructions.

2.7 Cultivation of *Plasmodium* spp.

Malaria infected blood sample was collected into heparin tubes. Malaria parasites were cultured in *McCoy's* 5A *medium* for *P. vivax* and RPMI-1640 medium for *P. falciparum* supplemented with 0.5% Albumax (Thermo Fisher Scientific, USA) according to standard *in vitro* culture techniques [22].

2.8 Immunofluorescence Assay (IFA)

IFA analysis was performed as described [23]. Briefly, thin blood smears were air-dried, fixed with 4% paraformaldehyde, and permeabilized with 0.1% triton-X 100. Smears were blocked with blocking buffer and stained with primary antibody (Rabbit anti-*Pf*ROM1 peptide pAb, Rabbit anti-*Pb*ROM1mAb and Rabbit anti-*Drosophila melanogaster* ROM1 pAb) overnight. After washing 3 times with PBS, Smears were then incubated with secondary antibody for 2 hours and mounted with coverslips and fluoromount mounting medium (Fritz Hoffmann-La Roche, Switzerland). Smears were then taken throughout one asexual life cycle (every 6 hours until 24 hours, then every 4 hours). Fluorescence images were taken by Nikon eclipse 80i.

3. RESULTS AND DISCUSSION

3.1 The Malaria Species Infected Red Blood Cell

According to the microscopic examination, the malaria species of 16 out of 30 samples were identified as *P. vivax*, 10 as *P. falciparum*, and 1 as a mixed infection of *P. vivax*/*P. falciparum*, while another 3 samples were negative. For 30 cases, multiplex PCR identified 10 as *P. falciparum*, 1 as a mixed infection of *P. vivax*/*P. falciparum* but 17 as *P. vivax* and 2 as negative. The gel picture for multiplex speciation PCR was shown in Figure 1.

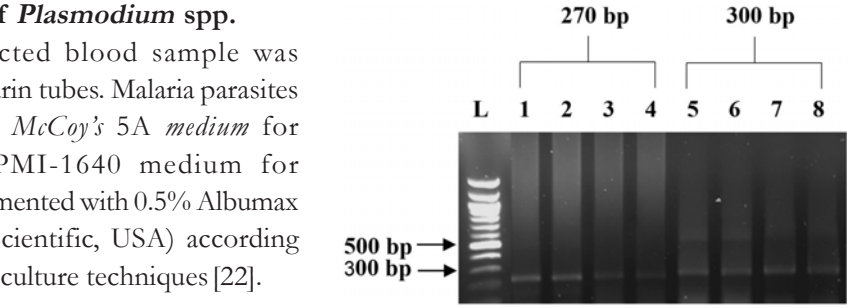

Figure 1. The positive bands of *P. falciparum* and *P. vivax* by using Multiplex PCR (Lane L; DNA ladder 100 bp, Lane 1-4: *P. falciparum* (270 bp), Lane 5-8 *P. vivax* (300 bp).

The Multiplex PCR detection of malaria is advantageous to microscopic detection that Multiplex PCR is more sensitive, specific and accurate [24] than microscopic technique, conventional methods, but it is time-consuming and expensive technique.

3.2 Molecular Analysis of *Pvrom*-like 1 Genes

The *Pvrom*-like 1 gene was successfully amplified from extracted RNA of all 17 *P. vivax*-infected blood samples and a ~1,268

bp band was visualized on an agarose gel (Figure 2). The 1,268-bp (GenBank acc. no. XM_001615211) of *Pvrom*-like 1 gene, including the 5'UTR (437 nt) and coding regions (831 nt).

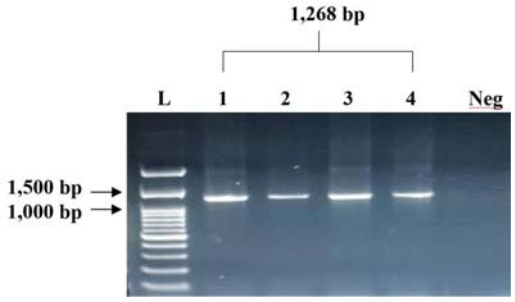

Figure 2. Detection of the *Pvrom*-like 1 gene using RT-PCR assay. (Lane L; 100 bp DNA Ladder, Lane 1-4 *Pvrom*-like 1 genes from *P. vivax* infected blood samples (1,268bp), Lane Neg; Negative control.

Nucleotide sequences in the *Pvrom1* genes from 17 *P.vivax* samples from malaria clinics in Maesod, Tak, Thailand were compared with the GenBank reference (acc. no.XM_001615211). SNPs were presented in 3 Tak samples adding to a total of 5 polymorphic sites. These have resulted in changes in deduced amino acid sequences of 3 Tak *Pvrom1* samples [A8T (2 samples), D13E, H219R, M240R (2 samples) and M250L] as shown in Figure 3.

```
PvROM1(REF)      MSNIHTLAEYRDDYGENLPFNRKYYQSQSSFIQRSKPIDVVNLIFPHFTWKSFIMAVSII 60
Tak4             .......T..................................................... 60
TAK15            .......T....E................................................ 60
                 *******:****:*********************************************

PvROM1(REF) 181  LILLWHVIRHRERVVFNIIFFSLISFFYYFTFNGSNIDHVGHLGGLLSGISMGILYNSQM 240
TAK2        181  ............................................................R 240
TAK4        181  .....................................R..................... 240
TAK7        181  ............................................................R 240
                 ************************************:*******************:

PvROM1(REF) 241  ENKPSWYDHMKMASYACLALLAIVPPIVLFAVPRTC 276
Tak4        241  .........L.......................... 276
                 *********:**************************
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Figure 3. Amino acid sequence alignment of the nucleotide sequences in coding region of *Pvrom1* gene exon 1-4 of blood-infected with *P. vivax* in malaria clinics in Maesod, Tak, Thailand with *Plasmodium vivax* SaI-1 rhomboid-like protease 1 partial mRNA (Acc. no.XM_001615211) in GenBank of NCBI.

The *Pvrom*-like 1 sequence was confirmed to consist of four exons, three introns and 437 bp of the non-coding region with a full-length of 1,891 bp (coding regions 831 bp) encoding a 276-aa protein (GenBank acc. no. XM_001615211). Contrary, The *Pfrom1* sequence was confirmed to consist of four exons and five introns (no found non-coding region) with a full-length of 1,569 bp (coding regions 837 bp) encoding for a 278-aa (GenBank acc. no. EU_180604.1) as shown in Figure 4 However, The result of ClastalW2 program shown that *Pvrom*-like 1 to *Pfrom1* have amino acid sequence similarity 85.14% as shown in Figure 5.

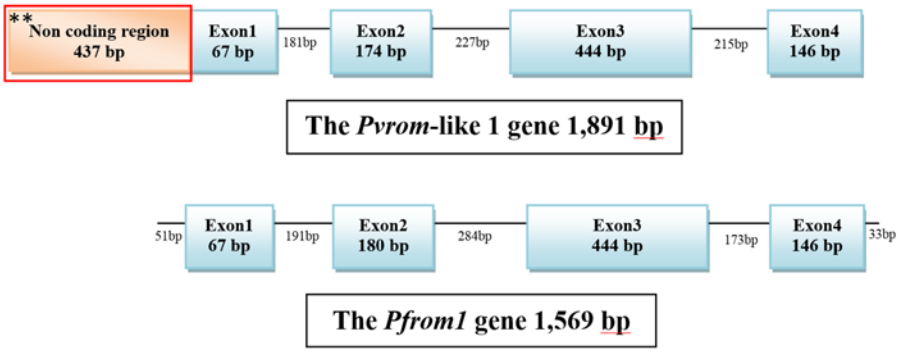

Figure 4. The full-length of the *Pvrom*-like 1 compare with *Pfrom1* genes (The *Pvrom*-like 1 sequence was confirmed to consist of four exons, three introns and 437 bp of the non-coding region** with a full-length of 1,891. Contrary, The *Pfrom1* sequence was confirmed to consist of four exons and five introns (no found non-coding region)).

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PvROM1      MSNIHTLAEYRDDYGENLPFNR--KYYQSQSSFIQRSKPIDVVNLIFPHFTWKSFIMAVS
PfROM1      MSNIHTLAEYRDDYAENTPFNRTPDYYQSQSSFVQRSKPIDLLNLIFPHFTWKSFMVVIS
PbROM1      MSNIHTLAEYRDGYGENLPFNRTPGYYASQSSFIQRSKPIDVVNLIFPNFTWKSFVMVVS
            ************.*.** ****   ** *****:*******::*****:******::.:*

PvROM1      IIQIIVFIISVSIKPADFLTPSDSLLITLGANVASRIKQGEIHRLILPIFLHANIFHTFF
PfROM1      IIQLIVFIISVSIKPADFLTPSDSLLVTLGANVASRIKQGEIHRLILPIFLHANIFHAIF
PbROM1      IIQIIVFIISLSISPGEFLTPSGSILVTLGANVGSKIKNGEIHRLILPIFLHANIFHTFF
            ***:******:**.*.:*****.*:*:******.*:**:******************::*

PvROM1      NVFFQLRMGFTLEKNYGIVKVIILYFLTGIYGNILSSSVTYCPIKVGASTSGMGLLGVVT
PfROM1      NIFFQLRMGFTLEKNYGIMKIIILYFVTGMYGNILSSSITYCPIKVGASTSGMGLVGIVT
PbROM1      NVFFQLRMGFTLEKNYGILKVAILYFVSGIYGNILSSSVTYCTIKVGASTSGMGILGIVA
            *:*****************:*: ****::*:********:*** ***********::*:*:

PvROM1      SELILLWHVIRHRERVVFNIIFFSLISFFYYFTFNGSNIDHVGHLGGLLSGISMGILYNS
PfROM1      SELILLWHIIRHRERVVFNIIFFSLISFFYYFTFNGSNIDHVGHLGGLISGISLGILYNE
PbROM1      SELILLWHIIRHRERVVFNILFFTLISILYYFTFNGSNIDHIGHLGGLLSGISIGMLYNE
            ********:***********:**:***::************:******:****:*:***.

PvROM1      QMENKPSWYDHMKMASYACLALLAIVPPIVLFAVPRTC
PfROM1      HMDNKPRWYNHLKIGSYISLVLLAIIPTVVLFTIPRTC
PbROM1      KMENKPTWYNNVKIASYACLVLLAIVPTVVLFAVPRAC

            :*:*** **:::*:.** .*.****:* :***::**:*
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```
Percent Identity  Matrix - created by Clustal2.1

          PbROM1     100.00   84.42   80.22
          PvROM1      84.42  100.00   85.14
          PfROM1      80.22   85.14  100.00
```

Figure 5. Amino acid sequence alignment of the PfROM1and PbROM1 compare with PvROM-like 1 protease.

3.3 Detection of PvROM-like 1 Protein with Anti-PfROM1 Peptide

The purified of recombinant PvROM-like 1 was separated by SDS-PAGE. The protein of interest can be detected by subsequent western blotting using anti-PfROM1 peptide antibody. The apparent molecular weight of PvROM-like 1 was estimated to be ~30 kDa in Lane 1, Recombinant PvROM-like 1, but it was not present in Lane Neg, negative control (Figure 6).

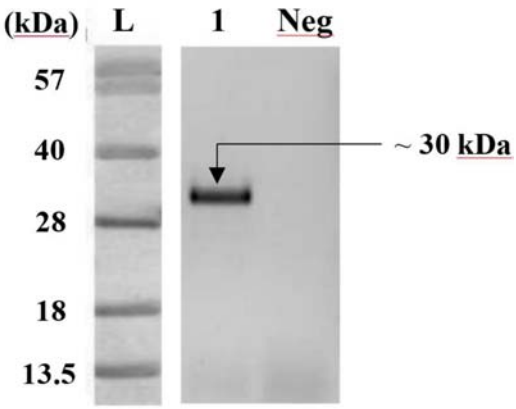

Figure 6. Western blots of PvROM-like 1 proteins separated by SDS- PAGE. (Lane L: protein marker, Lane 1: Recombinant PvROM-like 1 (~30 kDa), Lane Neg: negative control.

3.4 ROM-like 1 Protein is Expressed in All Erythrocytic Cycle

Our previous result showed that the PvROM-like 1 protein was successfully detected form anti-PfROM1 peptide thus rabbit anti-PfROM1 antibody was used to detect ROM-like 1 protein in *P. vivax* and ROM 1 in *P. falciparum*. To determine the presence of ROM1 protein, immunofluorescence assays (IFA) was performed with *P. vivax* and *P. falciparum* smeared on slides. Rabbit antibody against PfROM1 peptide showed staining of PfROM1 and PvROM-like 1 protein in malaria-infected blood, but no staining was detected in normal blood (Figure 7 upper panel). Moreover, PfROM1 and PvROM-like 1 were also detected by Rabbit anti-PbROM1 mAb (Figure 7 middle panel), but not anti-DroROM1 Ab (Figure 7 lower panel).

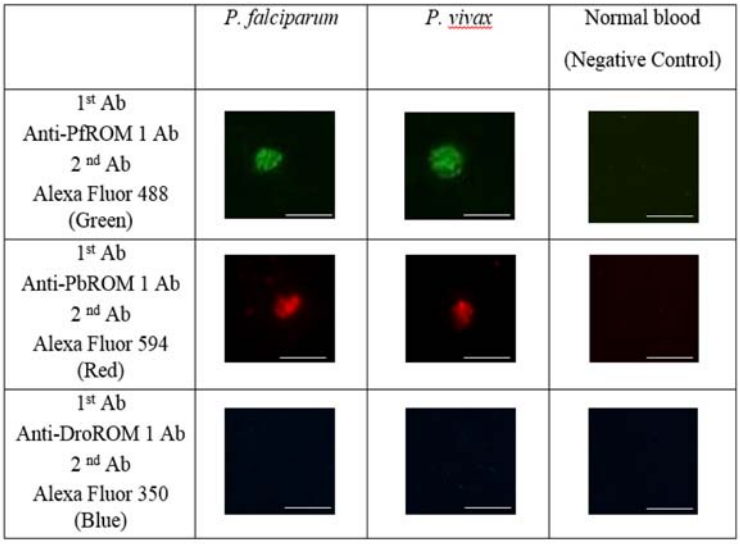

Figure 7. Detection of PvROM-like 1 and PfROM1 proteins in erythrocytic stage by IFA technique (Scale bars, 5 m).

PvROM-like 1 and PfROM1 in blood samples could be detected by anti-PfROM1 Ab and anti-PbROM1 Ab at dilution 1:200. Contrary, PvROM-like 1 and PfROM1 in blood samples could not be detected by anti-DroROM1 Ab and in normal blood using for negative control. As the limitation of producing antibody to PvROM-like 1 such as time consuming, costs of producing, the animal ethics, we used the anti-PfROM1

and anti-PbROM1 instead of anti-PvROM-like 1 Ab because the PvROM-like 1 and PfROM1 proteins can be detected by anti-PfROM1 Ab and anti-PbROM1 Ab, in which more than 80% amino acid sequence of the immunogen (Figure 5) of those antibodies was similar to samples so those antibodies can be used. In contrast, *Drosophila melanogaster* ROM1 pAb used for negative control.

To determine which stage of the malaria parasite that express ROM1 protein, *P. vivax* and *P. falciparum* were sampled at 0, 6, 12, 18, 24, 28, 32, 36, 48 h through an asexual life cycle in malaria culture and PvROM-like 1 and PfROM1 proteins were determined by IFA using Rabbit anti-*Pf*ROM1 peptide pAb, Rabbit anti-*Pb*ROM1mAb and Rabbit anti-*Drosophila melanogaster* ROM1 pAb. The results showed that PvROM-like 1 and PfROM1 proteins were initially found in the erythrocytic cycle at < 24h (rings-stage) in *P. vivax* and *P. falciparum,* which were presented 70% (7/10) and 80% (8/10) of total samples, respectively; at 28h (early trophozoites) in *P. vivax and P. falciparum* were presented 90% (9/10) and 100% (10/10), respectively; >28h (mid. trophozoites to late schizonts) in both *P. vivax* and *P. falciparum* were presented 100% in all samples (Table 1). PvROM-like 1 and PfROM1 proteins were also determined by IFA using Rabbit anti-PbROM1 mAb (Table 1).

Table 1. The number of ROM1 proteins in the erythrocytic stage of *P. vivax* and *P. falciparum*.

Antibodies / Sample	PfROM1 Ab (%)	PbROM1 Ab (%)	DroROM1 Ab (%)
***P. vivax* (time/stage)**			
0 h	0/10 (0)	0/10 (0)	0/10 (0)
6 h (Tiny rings)	2/10 (20)	2/10 (20)	0/10 (0)
12 h (Small rings)	5/10 (50)	5/10 (50)	0/10 (0)
24 h (Large rings)	7/10 (70)	7/10 (70)	0/10 (0)
28 h (Early trophozoites)	9/10 (90)	8/10 (80)	0/10 (0)
32 h (Mid. trophozoites)	10/10 (100)	10/10 (100)	0/10 (0)
36 h (Late trophozoites)	10/10 (100)	10/10 (100)	0/10 (0)
44 h (Schizonts)	10/10 (100)	10/10 (100)	0/10 (0)
48 h (Late schizonts)	10/10 (100)	10/10 (100)	0/10 (0)
***P. falciparum* (time/stage)**			
0 h	0/10 (0)	0/10 (0)	0/10 (0)
6 h (Tiny rings)	3/10 (30)	3/10 (30)	0/10 (0)
12 h (Small rings)	7/10 (70)	5/10 (50)	0/10 (0)
24 h (Large rings)	8/10 (80)	8/10 (80)	0/10 (0)
28 h (Early trophozoites)	10/10 (100)	9/10 (90)	0/10 (0)
32 h (Mid. trophozoites)	10/10 (100)	10/10 (100)	0/10 (0)
36 h (Late trophozoites)	10/10 (100)	10/10 (100)	0/10 (0)
44 h (Schizonts)	10/10 (100)	10/10 (100)	0/10 (0)
48 h (Late schizonts)	10/10 (100)	10/10 (100)	0/10 (0)

The ROM1 proteins were not found in the tiny ring stage at <6 hour which may be possible that quantity of PvROM-like 1 and PfROM1 was poorly developed in these stages that could not be directly detected by IFA. However, the ROM1 expression might be detectable by the more sensitive PCR technique, this method is based on the detection of nucleic acid sequences which will specific to *Plasmodium spp.* unless in a low quantity of PvROM-like 1 and PfROM1 samples.

The sample of immunofluorescence assay (IFA) is malarial infected RBC in cultures that culturing start from inoculating patient infected blood into culture medium until the malaria was grown at 48 h after the invasion. Although mixed stages of malaria were found in the samples, the presence of PvROM-like 1 and PfROM1 were clearly detected e" tiny rings by IFA in contrast to a previous study that PfROM1 was only studied in ≥ mature schizont (8 nuclei) [16].

The present study is the first known report on *P. vivax* rhomboid- like proteins in the erythrocytic cycle of the parasite. Characterization of Rhomboid protease 1 in *Plasmodium* spp. is not extensive. However, *Plasmodium* rhomboid 1 has been partially characterized in *P. berghei* [13], *P. yoelii* [14] and *P. falciparum* [15-16].

The previous studies of apicomplexa rhomboids including *Toxoplasma gondii* ROM1-5 (TgROM1-5) [10-12, 26-28], *Plasmodium falciparum* ROM4 (PfROM4) [6, 15] and *P. falciparum* ROM1 (PfROM1) proteins [6, 13, 15] were found to be an important organelle in the erythrocyte invasion. Moreover, the recent study in Thailand, we had studied the genetic diversity of malaria parasite by analyzing single-nucleotide polymorphisms (SNPs) of the *P. vivax* rhomboid-like protease 1 gene (*Pvrom1*) in parasites. The results reported the detection in PvROM-like 1 and PfROM1 proteases using the different clone of antibodies that can cross react with our protein of interest (29). Therefore, future studies could focus on the specific localization of PvROM-like1 protease in erythrocytic stage, and could determine its function whether it is an organelle that play an important role in cell invasion. Moreover, the study of function and mechanism of PvROM-like1 in *P. vivax* will help us to lay the groundwork for development of basic knowledge in this critical step in the malaria life cycle.

4. CONCLUSIONS

In summary, the previous studies of apicomplexa rhomboids were found to be an important organelle in the erythrocyte invasion [6, 10-13, 15, 26-28]. Our results demonstrated that PvROM-like 1 protein was found in *P. vivax* at all erythrocytic stages of the malaria life cycle, but the localization and its function has not been clearly characterized yet. However, the amino acid sequence of PvROM-like 1 and PfROM1 are also highly similar (85.14%) suggesting that PvROM-like 1 may potentially function similar to that of other apicomplexa parasites in the invasion of malaria into red blood cells. Therefore, the finding of PvROM-like 1 would be the target protein that essential for development of vaccine. Eventually, it will help us to find better way to prevent and protect the malaria infection.

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