# Mass Spectrometry Based-Proteomics Facilitates Interrogation of Toll and Imd signaling Pathways of *Anopheles dirus* Against *Plasmodium vivax* Infection

# การวิเคราะห์ด้วยโปรติโอมิกส์ชนิดอาศัยแมสสเปคโทรเมทรีเพื่อช่วยสืบหาวิถีสัญญาณของ Toll และ Imd ของยุงก้นปล่องชนิดไดรัสเพื่อต้านทานการติดเชื้อพลาสโมเดียม ไวแวกซ์

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**ABSTRACT**

Studying on interaction between mosquito and parasites elucidates defensive immunity mechanism against *Plasmodium* infection lead to find the way to prevent malaria transmission. Our study present innate immune signaling of *Anopheles dirus* has been induced in order to defense against *Plasmodium* infection. Toll and Imd pathway which are responsible for control immune effector genes, was investigated in this study with modern ESI-QTOF and bioinformatics. Midgut membrane protein of *An. dirus* infected *P. vivax* was analyzed by mass-spectrometry based proteomics. MS data were search by Trans-Proteomics Pipeline (TPP) platform with using Comet algorithm then perform validation of search result and finally quantification by XPRESS algorithm. The result revealed that infection of *P. vivax* stimulated *Plasmodium* effector for defensive function by Toll and Imd pathways and also up-regulated C-type lectin family as well.

**บทคัดย่อ**

การศึกษาปฏิสัมพันธ์ระหว่างยุงและปรสิตทำให้เข้าใจถึงกลไกการป้องกันด้วยระบบภูมิคุ้มกันเชื้อพลาสโมเดียม นำไปสู่การค้นหาวิธีป้องกันการแพร่โรคมาลาเรีย โดยการศึกษาในครั้งนี้เกี่ยวกับการส่งสัญญาณของระบบภูมิคุ้มกันโดยกำเนิดของยุงก้นปล่องสายพันธุ์ *Anopheles dirus* ที่ปกป้องจากการติดเชื้อ *Plasmodium vivax* โดยการศึกษานี้ใช้วิธีทางโปรติโอมิกส์แบบการใช้แมสสเปคโทรเมทรีเพื่อศึกษาโปรตีนในวิถี Toll และ Imd ซึ่งรับหน้าที่ในการควบคุมยีนที่แสดงออกด้านภูมิคุ้มกัน โปรตีนเยื่อหุ้มเซลล์จากกระเพาะส่วนกลางของยุง *An. dirus* ที่ติดเชื้อ *P. vivax* จะถูกนำมาวิเคราะห์โดยอาศัยแมสสเปคโทรเมทรี โดยผลข้อมูลจะถูกนำมาสืบค้นหาโปรตีนโดยโปรแกรม Trans-Proteomics Pipeline (TPP) ด้วย Comet อัลกอริธึ่มและคำนวณหาปริมาณโปรตีนด้วย XPRESS อัลกอริธึ่ม ผลการทดลองแสดงโปรตีนในวิถี Toll และ Imd มีเพิ่มขึ้นรวมถึงโปรตีนในกลุ่ม C-Type Lectin ด้วย

**Keywords:** *Anopheles dirus*, Malaria, Proteomics

**คำสำคัญ:** ยุงก้นปล่องชนิดไดรัส มาลาเรีย โปรติโอมิคส์

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**Introduction**

Malaria is the most devastating mosqutio-borne disease. It was estimated that the prevalence of malaria is approximately 216 million cases and mortality is more than 400,000 cases in 2016 worldwide. In addition, human populations in Southeast Asia, particularly Thailand, are at risk because of living in the presence of mosquito area. Although the death of malaria patients in Thailand was decreased, the risk factors, such as vector resistance, increasing of mosquito population caused by global climate change including malaria drug resistance remain concerning (WHO, 2017). Apparently, *An. gambiae*, the malarial vector in Africa have been studied intensively, particularly for malaria elimination campaign. Actually, *An. dirus* is primary vector in Thailand rather than *An. gambiae* and the study of role malaria infection in this species seem to be little. Thereby, *An. dirus*, is a vector that is susceptible to *P. vivax* which this type has the strong anthropophilic behavior that are hurdle to stop transmission of malaria in Thailand.

*Plasmodium vivax* distributes in broad geographical range, including Asia, particularly in Thailand. Additionally, *P. vivax* is highly proportional comparing to other *Plasmodium* species in Thailand. Malaria parasites are usually able to develop in both of human and mosquito host, which can be defined as intrinsic host and extrinsic host, respectively.

Studying involved mosquito activate immune signaling against *Plasmodium* mostly was conducted innate immune protein of *Drosophila*. Immune response regulate anti-Plasmodium have been found three major pathways included Toll, Immune deficiency (Imd) and (JAK/STAT) Janus kinase-signal transducers and activator of transcription. STAT pathway in *An. gambiae* infected by *P. falciparum* (Gupta et al., 2009) suggested that silencing of SOCS, STAT inhibitor lead to reduction of oocyst. The most commonly pathway defense *Plasmodium* and antimicrobial are Toll and Imd pathway, hallmark of innate immunity in *Drosophila* response infection via recognition of pathogen-associated molecular pattern (PAMPs), these two signaling pathway have interact synergistically by some effector genes was expression by regulated of two pathway (Tanji et al., 2007). Imd pathway induced transcription factor *Rel2* both midgut and fat body enhanced antiparasistic action lead to expression of immune effector such as thioester-containing protein 1 (TEP1), fibrinogen immunolectin 9 (FBN9), and a leucine-rich repeat family member (LRRD7/APL2). In addition to Toll pathway effect in parasites defensive by activated transcription factor Rel1 that can be induced immune effector gene include *Cercropin 1*, *Defensin 1* and *Gambicin1* (Clayton et al., 2014). Because of *Plasmodium* against common controlled by Toll and Imd signaling pathways we will investigates the expression in *An. dirus* infected with *P. vivax* in early infection. Although there were a number of researches have conducted mosquitoes innate immune against *Plasmodium,* however, mostly, it was conducted in *An. gambiae* which is major vector in Africa, for example studied of innate immune of *An. gambiae* infected *P. berghei* and *P. yoelii* by gene knockdown (Mitri et al., 2009) host-parasite interaction between *An. dirus* and *P. vivax* that important in Thailand have not yet been clarified so far. Proteomics combined with bioinformatics is the effective approach for viewing on responsive immunity protein of *An. dirus*, which is uncompleted genome sequencing organism. Nevertheless, there was a study conduct on uncompleted sequencing genome mosquito, *An. stephensi*,

which applied proteomics for studying on fat body induced by blood meal present activation of innate immune response protein potential be transmission blocking vaccine (Kumar et al., 2017).

In present study, we seek for the responsive proteins in *An. dirus* midgut which could be promising proteins in defensive mechanism against *P. vivax* and be potential transmission blocking vaccine target. To study proteome profiling mass spectrometry was used to fulfill knowledge of immune expression dynamic in *An. dirus* infected with *P. vivax*. Trans-proteomic pipeline (TPP) was open source software to analysis MS/MS data developed by Seattle Proteome Center (SPC). The workflow can be applied to peptides identification, protein validation and quantification (Pedrioli, 2010).

**Objectives of the study**

This study aimed to apply label-free mass spectrometry based proteomics to preliminarily identify proteins involving to immune response *An. dirus* mosquito among blood feeding and *P. vivax* infection group. Preliminary data can be the guidance for further analysis of protein expression such as immunoblotting and ELISA. The immune response would be elucidated for malaria transmission mechanism during invasion in mosquito midgut.

**Methodology**

**Midgut membrane protein preparations**

Mosquito midgut tissue derived from dissection 30 females *An. dirus* midgut day 5-7 for naïve condition mosquitoes was feed on sugar, blood meal condition was dissected after mosquito feed on blood meal after engorge immediately, 18 hours and 24 hours after blood feeding. *P. vivax* infected condition mosquito was feed on human blood contained gametocyte by collecting same time interval as blood meal condition. Infection batch used in this experiment was higher than 95% infection by sampling dissection to count oocyst and sporozoite. Midgut tissue was discarded blood by phosphate buffer then keep in phosphate buffer with protease inhibitor. The tissue was grinded to homogenate then centrifuge at 13,000 rpm, 4ºC for 15 minutes the supernatant was collected as soluble protein. The midgut pellet then was lysis with 10% SDS then grind and centrifuge at 13,000 rpm, 4 ºC for 15 minutes again to collect supernatant as midgut membrane protein.

**Protein separation by gel electrophoresis**

Forty µg of midgut membrane protein from each condition was separated by gel electrophoresis. Gradient 4-12% NU-PAGE gel was used with MOPS running buffer. Protein extracted was separated by applied electricity at 120 volt for 90 minute. After finish, the gel was stained with coomassie blue. The gel was destained to eliminate the background besides protein bands before performed in-gel digestion.

**Peptides preparation by in-gel digestion**

After protein extracted were separated completely, protein gel were sliced in to 17 small gel pieces along molecular weight order bands from naive midgut and 0 h, 18 h and 24 h after normal and infection blood meal. Each gel pieces was destained with 25mM Ammonium bicarbonate in 50% methanol then removed the color then added

100% acetonitrile for prepare the peptide digestion. To reduce the disulfide bond, the gel was added 10 mM dithiothreitol in 10 mM ammonium bicarbonate then incubated at 56 ºC for 1 h and removed. To alkylate was applied adding 100 mM Iodoacetamide in 10 mM ammonium bicarbonate and kept in dark at room temperature for 1 h. Then the gel was washed again with 100% acetonitrile twice. Then protein was digested by protease that cleavage specificity, 10 ng/µl trypsin was added into gel plug at room temperature for 20 minutes then incubated at 37 ºC overnight to allow enzyme cleavage. We extracted the peptide production using 50% acetonitrile in 0.1% trifluoroacetic acid at room temperature for 10 minutes. Then the extracted solution was dried in 37 ºC incubator for overnight. Finally kept the samples at -80 ºC until mass spectrometry analysis (Shevchenko et al., 2006).

**Bioinformatics analysis for *An. dirus* innate immune against *P. vivax***

The midgut protein samples were analyses by LC-MS/MS (Thermo scientific). Raw data was converted to MZML file by TPP tool then peptides search through Comet search engine. Database constructed from vectorbase database, which are orthologous with *An. gambiae* include two cascaded of signaling pathway, Toll and IMD pathway. Protein validation and quantification were conducted by peptide analysis workflow that include iProphet analysis to validate protein and XPRESS values determined protein level.

**Statistical analysis**

Protein validation was filtered by pass probability minimum at 0.95. Level of protein expression in each condition was normalized by level of protein expression of naïve condition then the ratio of protein expression level were log2 taken to classify expression level of protein.

**Results**

Protein identification was derived by MS/MS spectrum searching with *An. gambiae*. Peptides sequences that assigned with database, search were analyzed with PeptideProhet. The confidence of searching results were performed by filtering probability with TPP algorithm. Abundance of protein was show as XPRESS ratio of ion extracted. Protein-level of Imd pathway and Toll pathway was plotted as graph of log2 XPRESS ratio by compared Protein level between infection and blood meal (Fig. 1). Time interval was concerned because involved in parasite motile and development. The experiment observed from immediately intension (0 h), ookinete development (18 h) and motile ookinete (24 h). The result of Toll cascade revealed that Cact protein normally expression all time. Cec1 was up-regulated at 0 h post infected blood meal feeding mosquito. Pelle and Defensin family include; DEF2, DEF3 through Toll pathway and up-regulated ALP1A, ALP1C and TEP1 through Imd pathway. Mosquitoes activated effector protein; DEF2, TEP1, Ankyrin at 18 h. Proteins were up-regulated all time including, REL2, APL1A and DEF1-4 were up-regulated. Immune response to infection in Imd pathway included C-type lectin (CTLs), which member depend on specific mono or oligosaccharides biding site. Proteins CTL8, CTLSE1 were up-regulated at all interval time. CTL5, CTL9, CTL10 and CTLMA6 were responsible to defense *P. vivax* infection at between 18 and 24 h after infection. CTLSE2 and CTL3 and CTL4 were up-regulated at 0 h. CTL1 and CTLMA3 are up-regulated at 18 h. CTLMA6-9 were up-regulated at 24 h. The significantly different level response in 0 h infected were APL1C but not present in blood meal, CTL4 that up-regulated 3.1 folds from blood meal and at 24 h found REL2 and CTL9

up-regulated 5.7 and 9.6 folds respectively and CTL7 that high level (3.03) in infection but not present in blood meal. For Toll pathway DEF2 and DEF4 were up-regulated 2 fold at 0 h and 24 h, respectively (Table 1).

Protein expression in both pathways can be draw referred to information of *An. gambiae* mosquito (http://www.genome.jp/kegg). The illustration showed the cascade of Toll and Imd pathways (Fig.2).

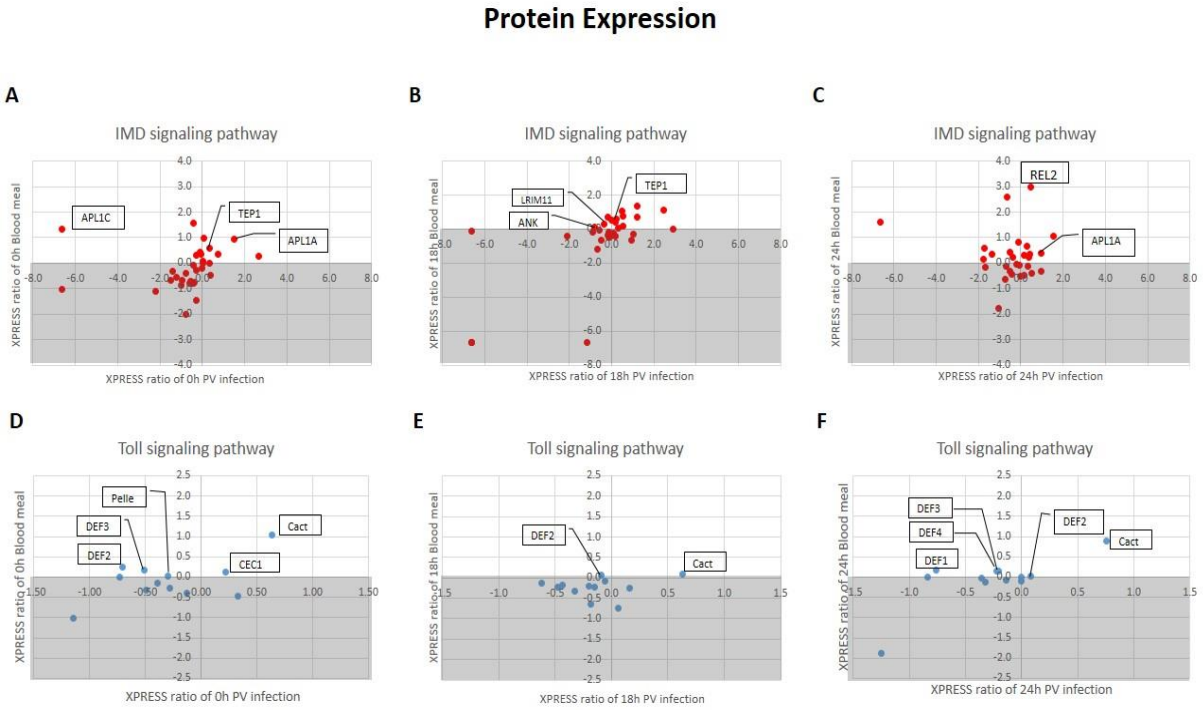


**Figure 1** Protein-level as log 2 XPRESS ratio was plotted. Protein response in infection mosquito placed in quadrant 1 and 4 of graph by Q1 have high level in both blood meal and infection, Q2 have overexpression in infection mosquito. A-C: protein expression in Imd signaling cascade at 0 h, 18 h and 24 h, respectively. D-F: protein expression in Toll signaling cascade at 0 h, 18 h and 24 h, respectively. Protein in box referred to Toll and Imd signaling cascade that up-regulate when infected.

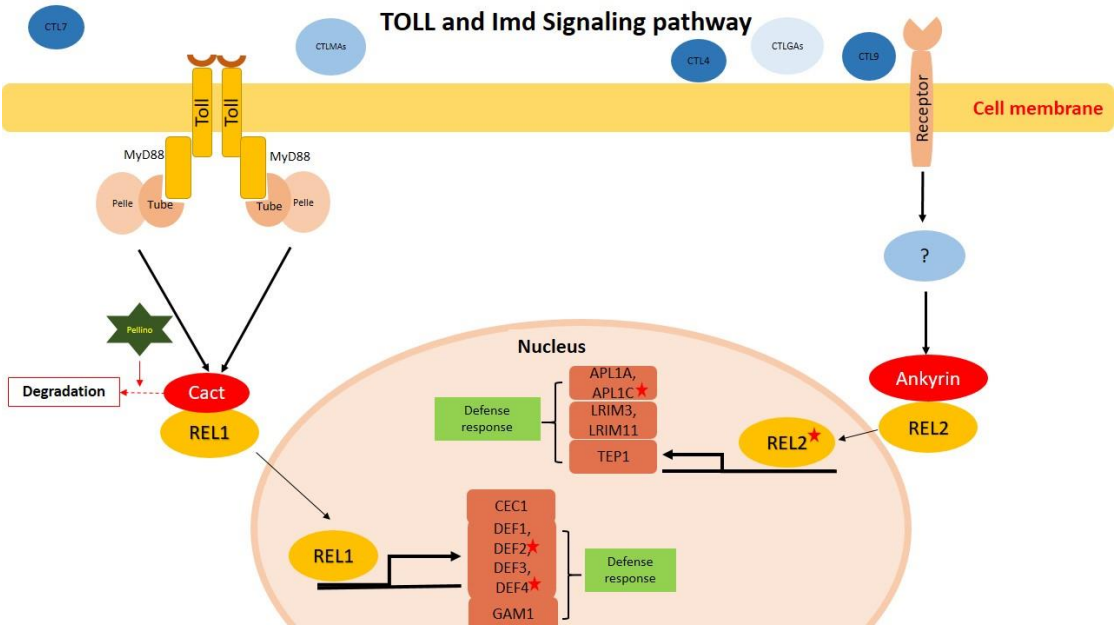


**Figure 2** Toll is transmembrane receptor activate Pelle and Tube then signal transduction to activate and translocate of REL1 transcription factor nucleus result in expression of defense response. Signal transduction of Imd result in REL2 transcription factor translocate and express effector gene for defense response include *APL1, LRIM, TEP1*. Star indicated protein have been identified by this proteomics approach, i.e. , APL1C and REL2 from Imd signaling pathway and DEF2, DEF3 that from Toll pathway, these protein have high up-regulated more than two folds in infection comparing to no infection.

**Table 1** Protein form Toll and Imd pathways and C-type lectin family identified by TPP tools using comet search with XPRESS ratio of blood meal and *P. vivax* infection that normalized by naïve mosquito

| Accession | Toll pathway<br>Protein description | XPRESS | | | | | |
|---|---|---|---|---|---|---|---|
| | | Blood meal condition | | | Infection condition | | |
| | | 0 h | 18 h | 24 h | 0 h | 18 h | 24 h |
| AGAP002966-PA | PELLE: TOLL pathway signalling Ser/Thr kinase | 0.81 | 0.96 | 0.91 | 1.03 | 0.95 | 0.95 |
| AGAP003062-PA | TUBE: TOLL pathway signaling | 0.71 | 0.74 | 0.8 | 0.8 | 0.89 | 0.92 |
| AGAP004232-PA | Pellino | 0.82 | 1.04 | 0.78 | 0.84 | 0.6 | 0.98 |
| AGAP007938-PA | cact: protein cactus (TOLL pathway signalling) | 1.55 | 1.55 | 1.69 | 2.07 | 1.07 | 1.86 |
| AGAP009515-PA | REL1: TOLL pathway signalling NF-kappaB Relish-like transcription factor | 0.91 | 1.12 | 1 | 0.77 | 0.84 | 0.94 |
| AGAP011294-PA | DEF1: defensin anti-microbial peptide | 0.76 | 0.87 | 0.59 | 0.91 | 0.87 | 1.13 |

**Table 1 (cont.)** Protein form Toll and Imd pathways and C-type lectin family identified by TPP tools using comet search with XPRESS ratio of blood meal and *P. vivax* infection that normalized by naïve mosquito

| Accession | Toll pathway Protein description | XPRESS | | | | | |
|---|---|---|---|---|---|---|---|
| | | Blood meal condition | | | Infection condition | | |
| | | 0 h | 18 h | 24 h | 0 h | 18 h | 24 h |
| AGAP004632-PA | DEF2: defensin anti-microbial peptide | 0.61 | 0.94 | 1.06 | 1.2 | 1.04 | 1.02 |
| AGAP007199-PA | DEF3: defensin anti-microbial peptide | 0.7 | 0.9 | 0.87 | 1.14 | 0.86 | 1.11 |
| AGAP005416-PA | DEF4: defensin anti-microbial peptide | 1.25 | 0.72 | 0.86 | 0.73 | 0.85 | 1.11 |
| AGAP007200-PA | DEF5: defensin anti-microbial peptide | 0.45 | 0.65 | 0.42 | 0.5 | 0.91 | 0.27 |
| AGAP000693-PA | CEC1: cecropin anti-microbial peptide | 1.16 | 0.88 | 0.56 | 1.1 | 0.64 | 1 |
| AGAP008645-PA | GAM1: gambicin anti-microbial peptide | 0.6 | 0.8 | 1 | 1 | 0.8 | 1 |
| AGAP000929-PA | CTLSE1: C-Type Lectin (CTL) - selectin like | 0.93 | 1.43 | 1.35 | 1.33 | 1.11 | 1.28 |
| AGAP000123-PA | CTLSE2: C-type lectin (CTL) | 1.01 | 1.14 | 0.87 | 1.01 | 0.76 | 0.97 |
| AGAP004811-PA | CTL1: C-type lectin (CTL) | 0.81 | 1.16 | 0.47 | 0.36 | 1.53 | 0 |
| AGAP010709-PA | CTL2: C-type lectin (CTL) | 0.75 | 0.66 | 0.6 | 0.58 | 0.94 | 0.65 |
| AGAP004810-PA | CTL3: C-type lectin (CTL) | 1.06 | 2.04 | 1.53 | 1.98 | 0.83 | 0 |
| AGAP005335-PA | CTL4: C-type lectin (CTL) | 0.76 | 1.47 | 1.13 | 2.95 | 1.68 | 1.23 |
| AGAP000443-PA | CTL5: C-type lectin (CTL) | 0.98 | 1.26 | 1.22 | 0.86 | 1.06 | 1.58 |
| AGAP006267-PA | CTL6: C-type lectin (CTL) | 0.81 | 1.07 | 0.94 | 1.24 | 0.79 | 1.79 |
| AGAP000940-PA | CTL7: C-type lectin (CTL) | 6.44 | 0 | 0 | 1.21 | 0.93 | 3.03 |
| AGAP003625-PA | CTL8: C-type lectin (CTL) | 0.96 | 0.88 | 1.3 | 1.26 | 1.66 | 1.19 |
| AGAP002625-PB | CTL9: C-type lectin (CTL) | 0.36 | 5.45 | 0.63 | 0.63 | 2.16 | 6.09 |
| AGAP009316-PA | CTL10: C-type lectin (CTL) | 1.29 | 2.33 | 2.9 | 0.71 | 2.57 | 2.09 |
| AGAP007411-PA | CTLMA1: C-Type Lectin (CTL) - mannose binding | 0 | 0.9 | 0.61 | 0.29 | 0.73 | 0.91 |
| AGAP005334-PA | CTLMA2: C-Type Lectin (CTL) - mannose binding | 0.75 | 0 | 0.64 | 0.94 | 0 | 0 |
| AGAP007412-PA | CTLMA3: C-Type Lectin (CTL) - mannose binding | 0.5 | 2.33 | 0.72 | 0.55 | 1.61 | 0 |
| AGAP007407-PA | CTLMA4: C-Type Lectin (CTL) - mannose binding | 0.22 | 0.54 | 0.69 | 0.47 | 0.87 | 0.81 |
| AGAP007410-PA | CTLMA5: C-Type Lectin (CTL) - mannose binding | 0.01 | 0 | 0.48 | 0.49 | 0 | 0.29 |
| AGAP005332-PA | CTLMA6: C-Type Lectin (CTL) - mannose binding | 0.38 | 1 | 0.3 | 0.8 | 1.42 | 1.52 |

**Table 1 (cont.)** Protein form Toll and Imd pathways and C-type lectin family identified by TPP tools using comet search with XPRESS ratio of blood meal and *P. vivax* infection that normalized by naïve mosquito

| Accession | Imd pathway Protein description | XPRESS | | | | | |
|---|---|---|---|---|---|---|---|
| | | Blood meal condition | | | Infection condition | | |
| | | 0 h | 18 h | 24 h | 0 h | 18 h | 24 h |
| AGAP005332-PB | CTLMA6: C-Type Lectin (CTL) - mannose binding | 0.822 | 1.06 | 0.71 | 0.82 | 0.86 | 1.34 |
| AGAP010708-PA | CTLMA7: C-Type Lectin (CTL) - mannose binding | 0.52 | 0.62 | 0.39 | 0.62 | 0.45 | 1.29 |
| AGAP007408-PA | CTLMA8: C-Type Lectin (CTL) - mannose binding | 0.68 | 0.89 | 0.77 | 0.58 | 0.78 | 1.17 |
| AGAP002911-PA | CTLMA9: C-Type Lectin (CTL) - mannose binding | 0.59 | 0.45 | 0.29 | 0.76 | 0 | 1.12 |
| AGAP010196-PA | CTLGA1: C-Type Lectin (CTL) - galactose binding | 1.68 | 1.93 | 1.41 | 1.29 | 0.63 | 0.77 |
| AGAP006430-PB | CTLGA2: C-Type Lectin (CTL) - galactose binding | 0.66 | 0.23 | 0.75 | 0.58 | 0.75 | 0.73 |
| AGAP010193-PA | CTLGA3: C-Type Lectin (CTL) - galactose binding | 0.78 | 0.92 | 0.31 | 0.59 | 0.9 | 0.9 |
| AGAP028730-PA | Ankyrin | 1.02 | 0.57 | 1 | 1.04 | 1.09 | 0.7 |
| AGAP010815-PA | TEP1: thioester-containing protein 1 | 1.27 | 1.15 | 1.25 | 1.49 | 1.31 | 0.92 |
| AGAP006747-PA | REL2: IMD pathway signalling NF-kappaB Relish-like transcription factor | 1.25 | 7.5 | 1.38 | 1.38 | 3.87 | 7.88 |
| AGAP007033-PA | APL1C: Anopheles Plasmodium-responsive Leucine-Rich Repeat 1C | 0 | 0 | 1.95 | 2.51 | 0 | 0.8 |
| AGAP007034-PA | LRIM11: leucine-rich immune protein (Short) | 0.59 | 0.78 | 0.95 | 0.25 | 1.22 | 0.94 |
| AGAP007035-PA | APL1B: Anopheles Plasmodium-responsive Leucine-Rich Repeat 1B | 0.43 | 0.95 | 0 | 0.68 | 0.71 | 0.5 |
| AGAP007036-PA | APL1A: Anopheles Plasmodium-responsive Leucine-Rich Repeat 1A | 2.8 | 1.4 | 1.92 | 1.92 | 2.12 | 1.32 |
| AGAP007037-PA | LRIM3: leucine-rich immune protein (Long) | 0.69 | 0.71 | 1.12 | 0.61 | 0.64 | 0.72 |

**Discussion**

Mosquito midgut membrane was response for nutrition uptake, diuresis and also barrier of toxic pathogen and parasite. The innate immunity required for eliminate *Plasmodium* involved parasite killing or melanization. Some of proteins in signaling pathway normally expression such as Cact, negative regulator of the nuclear localization for homeostatic balance (Govind et al., 1993). Leucine-rich repeat protein, LRIM and APL have function in parasite killing like TEP1 which overexpression in 0 h and 18 h may responsible for defense at early infection. Protein defensins have high level both blood meal and infection may effect on microbial or *Plasmodium* infection. C-type lectin is one of *Plasmodium* effector have function in insect immunity. Member of CTLs, CTLMAs and CTLMAs up-regulated all time interval mean these family regulated parasites with other innate immune. In addition, other mosquito defense involved detoxification may up-regulated by blood meal response for microbial infection and stress from blood meal similar to *Aedes aegypti* (Sanders et al., 2003) instead of only immune effector genes. Innate immune protein have originate from different location for example in fat body or hemolymph that may be involved in anti-*Plasmodium*. This studied found CTLs family in midgut membrane but it have been reported that CTLs were soluble protein secreted from hemolymph may because of it work for protect infection cell as well.

**Conclusion**

The result concluded that infectious blood meal triggered signaling pathway to defense response. Start on immediately infected at 0 h until 24 h, ookinete motile to midgut epithelial membrane. We found that at 0 h after infection activate APL1C from Imd pathway and DEF2 and DEF3 from Toll pathway. Mosquito response ookinete at 18 h after infection by up-regulation LRIM11 and ankyrin from Imd pathway DEF2 by Toll pathway. After infection 24 h DEF1, DEF3, DEF4 were up-regulated by Toll pathway. Moreover other innate immune molecules present up-regulated family of CTLs, CTLSEs and CTLMA.

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