รายงานวิจัยฉบับสมบูรณ์

โครงการ การพัฒนาของระยะมีเพศของเชื้อพลาสโมเดียมไวแวกซ์และความสัมพันธ์ของการพัฒนาของระยะมีเพศกับการติดเชื้อในยุง

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สัญญาเลขที่ TRG5880144

รายงานวิจัยฉบับสมบูรณ์

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รายละเอียดโครงการ

สัญญาเลขที่ TRG5880144

ชื่อโครงการ (ไทย) การพัฒนาของระยะมีเพศของเชื้อพลาสโมเดียมไวแวกซ์และความสัมพันธ์ของการพัฒนาของระยะมีเพศกับการติดเชื้อในยุง

ชื่อโครงการ (อังกฤษ) Development of *Plasmodium vivax* gametocyte and its association with mosquito infectivity

หัวหน้าโครงการ.....ดร.วัลลภา รูปสูง สังกัด คณะเวชศาสตร์เขตร้อน มหาวิทยาลัยมหิดล

ผู้ประกอบการผู้ร่วมทุน

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Abstract

Plasmodium vivax is the most widely distributed human malaria parasite and a major cause of morbidity of people outside Africa. One of the unique characteristics of this parasite is early gametocytogenesis which happened much earlier than other human malaria parasites. Most *P. vivax* patients are thus expected to carry gametocytes in their blood circulation before seeking treatment, allowing the parasites ample times to be transmitted. In the areas of stable malaria transmission, the villagers who have had multiple episodes of malaria seem to have a higher chance of carrying asymptomatic infection due to acquired immunity against the parasite. These infections, even at very low parasite density, have been shown to be infective to mosquitoes and therefore contribute to the reservoir of transmission. Although *P. vivax* infections in endemic areas are mostly asymptomatic and associated with very low parasite density called sub-microscopic carriers, they are deemed important for maintaining malaria transmission with unknown gametocytes and infectivity levels. The relative contribution to transmission of symptomatic and asymptomatic infected-*P. vivax* peoples is still unclear. We have developed a surrogate markers for detecting infective gametocytes and to demonstrate the transmissibility of *P. vivax* at low gametocyte density. Serial dilution of *P. vivax* infected blood from malaria patients was performed in this study as the presenter of very low density of *P. vivax*-gametocyte and used them in membrane feeding assays. The qPCR-based assay was use to quantify the expression level of the gametocyte specific markers, pvs25 and pvs16, from the diluted blood samples and relate to the infectivity in the mosquito. In this study, both *Pvs25* and *Pvs16* were successfully used as molecular markers for gametocyte detection in sub-microscopic carrier and as a surrogate molecular markers for infective gametocytes of *P. vivax*.

Keyword: *Plasmodium vivax*, Gametocytogenesis, Mosquito Infectivity, and Transmission blocking vaccine.

บทคัดย่อ

พลาสโมเดียมไวแวกซ์ (*P. vivax*) เป็นเป็นเชื้อมาลาเรียที่มีการแพร่กระจายมากที่สุดและเป็นสาเหตุสำคัญของการเจ็บป่วยของผู้คนที่อยู่นอกทวีปแอฟริกา ลักษณะเด่นที่สำคัญของเชื้อ *P. vivax* คือกระบวนการสร้างระยะสืบพันธุ์เกิดขึ้นเร็วกว่าเชื้อมาลาเรียชนิดอื่น โดยเชื้อสามารถสร้างระยะสืบพันธุ์ก่อนที่ผู้ติดเชื้อจะแสดงอาการป่วย ซึ่งทำให้การแพร่กระจายของเชื้อจากคนไปสู่ยุงเกิดขึ้นได้อย่างรวดเร็ว นอกจากนี้ในพื้นที่ที่มีระบาดของเชื้อมาลาเรียอย่างต่อเนื่องคงที่ ชาวบ้านที่ติดเชื้อซ้ำๆ จะมีโอกาศพัฒนาไปเป็นผู้ติดเชื้อที่ไม่แสดงอาการได้เนื่องจากมีการสร้างภูมิคุ้มกันขึ้นมา แม้การติดเชื้อส่วนใหญ่จะเป็นแบบไม่แสดงอาการและมีความหนาแน่นของเชื้อต่ำมาก (sub-microscopic carriers) แต่ถือว่ามีส่วนสำคัญในการแพร่กระจายของเชื้อมาลาเรียในพื้นที่ การจะบอกถึง contributionในการแพร่เชื้อจากคนไปสู่ยุงในกลุ่มผู้ป่วยแบบแสดงอาการและไม่แสดงอาการนั้นยังไม่มีวิธีที่ชัดเจน เราจึงได้พัฒนา Molecular markers สำหรับใช้ในการตรวจหาระยะสืบพันธุ์ของเชื้อ *P. vivax* โดยได้มีจำลองทำการเจือจางเชื้อให้มีปริมาณต่ำเพื่อ แล้วนำไปให้ยุงกินเลือดด้วยวิธี membrane feeding assay เพื่อแสดงให้เห็นถึงความสามารถในการแพร่ของเชื้อ P. vivax ที่มีปริมาณต่ำๆ และทำการทดสอบโดยใช้ qPCR ใช้เพื่อหาปริมาณระดับการแสดงออกของgenes ในระยะมีเพศ โดยการตรวจหา pvs25 และ pvs16 ในการศึกษานี้พบว่าทั้ง Pvs25 และ Pvs16 มีความไวสูงสามารถใช้เป็น Molecular markers ในการตรวจหาความสามารถในการแพร่ของเชื้อ *P. vivax* จากคนไปสู่ยุง

คำสำคัญ พลาสโมเดียมไวแวกซ์ กระบวนการสร้างระยะสืบพันธ์, การติดเชื้อในยุง และ วัคซีน

บทสรุปผู้บริหาร

Plasmodium vivax is the most widely distributed human malaria parasite and a major cause of morbidity of people outside Africa. One of the unique characteristics of this parasite is early gametocytogenesis which happened much earlier than other human malaria parasites. Most *P. vivax* patients are thus expected to carry gametocytes in their blood circulation before seeking treatment, allowing the parasites ample times to be transmitted. In the areas of stable malaria transmission, the villagers who have had multiple episodes of malaria seem to have a higher chance of carrying asymptomatic infection due to acquired immunity against the parasite. These infections, even at very low parasite density, have been shown to be infective to mosquitoes and therefore contribute to the reservoir of transmission. Although *P. vivax* infections in endemic areas are mostly asymptomatic and associated with very low parasite density called sub-microscopic carriers, they are deemed important for maintaining malaria transmission with unknown gametocytes and infectivity levels. The relative contribution to transmission of symptomatic and asymptomatic infected-*P. vivax* peoples is still unclear. We have developed a surrogate markers for detecting infective gametocytes and to demonstrate the transmissibility of *P. vivax* at low gametocyte density. Serial dilution of *P. vivax* infected blood from malaria patients was performed in this study as the presenter of very low density of *P. vivax*-gametocyte and used them in membrane feeding assays. The qPCR-based assay was use to quantify the expression level of the gametocyte specific markers, pvs25 and pvs16, from the diluted blood samples and relate to the infectivity in the mosquito. In this study, both *Pvs25* and *Pvs16* were successfully used as molecular markers for gametocyte detection in sub-microscopic carrier and as a surrogate molecular markers for infective gametocytes of *P. vivax*.

สารบัญ

บทที่ 1.บทนำ

1.1 ความเป็นมาของโครงการ

In the areas of stable malaria transmission, the villagers who have had multiple episodes of malaria seem to have a higher chance of carrying asymptomatic infection. These people have parasites in their blood circulation but do not feeling feverish and do not seek treatment. Asymptomatic infections of *P. vivax* are still common in the endemic areas of Thailand due to acquired immunity against the parasite. These infections, even at very low parasite density, have been shown to be infective to mosquitoes and therefore contribute to the reservoir of transmission. For malaria elimination programs, transmission-blocking vaccine (TBV) has recently regained attention from the scientific community as it has a potential to be a powerful and cost effective tool and to reduce disease transmission, especially in the areas where asymptomatic malaria are common. TBV seeks to stimulate antibodies in the human host which, when taken during the blood meal, interfere the development of the parasites inside the mosquito. The molecular targets of *P. vivax* TBV development has mostly been those on gametes or ookinetes. Targeting the development of gametocytes is an alternative that has been little explored.

Unlike gametocyte development in *P. falciparum*, that in *P. vivax* has not been well characterized. The development of P. vivax gametocyte morphology is unknown, as is when the gametocyte becomes competent for transmission. The information on *P. vivax* gametocyte development will give a better understanding of gametocyte biology and their infectiveness to the mosquito, allowing future identification of novel candidates for transmission blocking vaccine.

1.2 วัตถุประสงค์

In this study, we aim to characterize gametocytogenesis of *P. vivax* and identify gametocytemarkers related to mosquito infectivity.

1.3 แผนการดำเนินงานวิจัย

Period	**Experimental plan**
0-6 month	**Study gametocyte maturation of *P. vivax*** 1. Determine the infectivity of *P. vivax* gametocytes from *ex-vivo* culture by performing membrane feeding assay. 2. Phenotyping of *P. vivax* gametocytes by performing immunofluorescense staining using gametocytes specific antibodies.
7-12 month	Determine the transmissibility of *P. vivax* at low parasite density by membrane feeding assay 1. Perform membrane feeding assay on serially diluted *P. vivax* infected blood. 2. Collect the serially diluted *P. vivax* infected blood for qPCR analysis.
13-24 month	1. Perform membrane feeding assay on serially diluted *P. vivax* infected blood. 2. Collect the serially diluted *P. vivax* infected blood for qPCR analysis. 3. qPCR analysis of *P. viviax* infected blood. 4. Data analysis and manuscript preparation

บทที่ 2 Literature review

Plasmodium vivax is the most widely distributed human malaria parasite and a major cause of morbidity of people outside Africa. Gametocyte is an infectious stage of malaria parasites which is transmitted to mosquitoes via the blood meal. One of the major concerns on *P. vivax* infection is early gametocytogenesis which happens much earlier than other human malaria parasites. Most *P. vivax* patients are thus expected to carry infectious gametocytes in their blood circulation before seeking treatment, allowing the parasites ample times to be transmitted. Moreover the villagers who have had multiple episodes of malaria seem to have a higher chance of being asymptomatic, rendering estimation of infectious gametocyte reservoir at the community level difficult.

Asymptomatic infections of *P. vivax* are still common in endemic areas due to acquired immunity against the parasite. These infections, even at very low parasite density, have been shown to be infective to mosquitos and are therefore expected to contribute to the reservoir of transmission [1, 2]. With more sensitive techniques for detection of the parasite available, the prevalence of asymptomatic infection has been shown to be higher than previously expected [3]. Transmission-blocking vaccine (TBV) for malaria has recently regained attention from the scientific community. The idea behind TBV seeks to raise antibodies in the human host which, once taken during the blood meal, interfere the development of the parasites inside the mosquito. The molecular targets of TBV development has mostly been those on gametes or ookinetes [4, 5]. Targeting the development of gametocytes is an alternative that has been little explored. Studies of *P. falciparum* gametocytes have been far more advanced compared to *P. vivax* mainly due to the availability of the in vitro production of gametocytes [6, 7]. Gametocyte development in *P. falciparum* takes approximately 10-14 days. Gametocyte development of *P. falciparum* has been described in to five distinctive stages (stages I to V) which can be identified by Giemsa-stained blood smears, with an exception of stage I which is morphologically very similar to trophozoites [8]. The transition from stage I to stage II of P. falciparum gametocyte coincides with a change from a roundish shape to an elongated form with a distinctive pigment pattern which occupies half of the erythrocyte. From stage III onwards, gametocytes can be distinguished into female and male. Once they become more mature, there is a marked increase in sub-cellular organelles such as ribosome, Golgi complex, endoplasmic reticulum and mitochondria, which is reflected in the deeper blue staining of cytoplasm of macrogametocytes on Giemsa smear. Stage III gametocytes have an appearance of a rhomboid and become more symmetrical once they mature to stage IV. The pigment of both stages are still widely spread over the length of the parasite. Stage V is the mature stage of gametocytes. At this stage, gametocytes are bent with rounding ends and have centralized chromatin and pigment granules. A number of proteins have been identified to be gametocyte specific [9-11]. Several of them are important for gametocyte development and have been extensively characterized, including Pfs16, Pfs27, Pfs230, and sex specific Pfs47, Pfg377 and alpha tubulin II. Importantly, although Pfs16 is produced over the period of gametocytogenesis, other proteins seem to be more specific to a particular developmental stage [12-15]. For example, Pfg27 is expressed as early as 30-40 hr after merozoite invasion and has its peak expression at stage II before dropping to a barely detectable level in stage V [15]. The Pfg 27 has no orthologs in other human malaria parasites and may contribute to the longer period of gametocyte development of this parasite. Pfs230 starts to express in stage II, peaks at stage III and continues to express until mature stage V. It is one of the targets of transmission blocking vaccine [4, 16-17]. Pfg 377

expression is associated with the development of osmophilic bodies of gametocytes. Osmophillic bodies start to appear at the periphery of stage IV macrogametocyte and in rare occasion microgametocyte. The expression of Pfg377 in association with osmophillic bodies is progressively increased as macrogametocytes mature . The Pfs47 protein is found solely in macrogametocyte. The expression of this protein starts at stage IV onward [18]. While Pfs47 is macrogametocyte specific, alpha tubulin II is specific to microgametocytes [19]. Unlike P. falciparum, gametocyte development in *P. vivax* has not been well characterized. Nothing is known about the development of morphology, as is when a gametocyte becomes competent. The 3-day longevity of *P. vivax* gametocytes is also much shorter than that *P. falciparum* gametocytes. Gametocytogenesis of *P. vivax* occurs much earlier than *P. falciparum*. Most *P. vivax* patients are thus expected to carry gametocyte in their blood circulation before seeking for treatment. Fresh isolates of *P. vivax* are always asynchronous; so gametocytes obtained directly from a single patient are expected to vary in degree of maturation. This makes studies of gametocyte development of this parasite difficult.

Despite the lack of in vitro continuous culture of *P. vivax*, the recently developed humanized mouse model, in which immune-deficient mice engrafted with human hepatocytes were shown to support both liver and blood stage development [20-23], offers new possibilities to study *P. vivax* gametocytes. The information on gametocyte development will give a better understanding of the gametocyte biology and their infectiveness to the mosquito as well as the identification of novel candidates for transmission blocking vaccine

บทที่ 3 ระเบียบวิธีการวิจัย

3.1 Study location and *Plasmodium vivax* infected blood collection

The study was performed at Mahidol Vivax Research Unit (MVRU), Faculty of Tropical Medicine, Mahidol University. Laboratory reared female *Anopheles dirus* mosquitoes used in this study have been maintained at the insectaria facility at MVRU. Malaria infected blood samples were collected from patients attending themselves at malaria clinics in Tak province, North-western Thailand and Ubonratchathani province, North Eastern, Thailand. The Human Subjects protocol for this study was approved by the Ethical Review Committee of Faculty of Tropical Medicine, Mahidol University (MUTM 2011-040-05). After obtained inform consent, 20 ml of venous blood were collected in a heparinized tube. The infected blood was kept at all time in a warm box (37.0°C +/- 5°C) immediately after blood collection and during transportation to onsite laboratory. The maximum time from blood collection to mosquito feeding was 6 hours.

3.2 Blood preparation and membrane feeding assay

To assess the relationship between parasite density and mosquito infectivity, *P. vivax* infected blood was twofold serially diluted with 50% hematocrit (hct) naïve blood type O for 12 dilutions and fed to *An. dirus* mosquito. To prepared naïve O cell at 50%, whole blood type O obtained from Thai Red Cross was washed 3 times with cold RPMI 1640 incomplete medium and centrifuged at 800 xg for 10 minutes. Packed O cell was resuspended to 50% hct with non-inactivated naïve AB serum. The 50% hct O cells was kept at 4°C for not more than 2 weeks until used.

Plasmodium vivax infected blood was washed twice with warmed RMPI1640 incomplete medium. All centrifugation step was performed at 37°C under 800 xg for 10 minutes. Packed infected blood was resuspended with equal volume of non-inactivated naïve human AB serum to make 50% hct and this is considered as undiluted infected blood. The infected blood was then 2 fold serially diluted with 50% O cells for 12 dilutions with final volume of 600 µl. For each dilution, 50 µl of diluted blood was store in 200 µl of RNAprotect Cell Reagent® (Qiagen, Germany), mixed by vortexing and stored at -80°C for qPCR analysis. At each dilution, thick blood smear was prepared from 1 µL of diluted blood and stained with 10% Giemsa for 10 minutes for quantification of parasite and gametocyte density. The remaining diluted blood was fed to 100 female *An. dirus* mosquitoes for 30 minutes using established membrane feeding assay. The infectivity in the engorged mosquito was examined on day 7 post feeding by examining the presence of oocyst in the mosquito's midgut. Mosquito infections rate was expressed as infection percentage and oocyst intensity was expressed as the arithmetic mean of oocysts count per infected mosquito.

3.3 Nested PCR analysis

Nested PCR was performed to confirm *P. vivax* single infection. The genomic DNA was extracted from 50 µl of infected blood using QIAamp® DNA Mini Kit (Qiagen, Germany) according to manufacturer's instruction. Two rounds of PCR were performed to amplify 18S ribosomal RNA gene (18SrRNA) of *P. falciparum, P. vivax, P. ovalae, P. malariae, and P. knowlesi* using genus and species specific primer sets (table S1-S6) as described previously [24].

3.4 Extraction of RNA

RNA was extracted using the RNeasy® plus mini kit (Qiagen, Germany) following the manufacture′s procedures. The sample collected in RNAProtect was centrifuged at 5000 xg for 10 min to pellet down the RNA. After discarded the supernatant, 600 µL of RLT buffer was added into the pellet and mixed thoroughly for 30 sec using vortex. The sample was then loaded to a gDNA eliminator column. After washed the column with RW1 washing buffer, 80 µl of RNase-free DNase working solution was added and incubated for 15 minutes at room temperature. After washed the column with RW1 buffer, RNA was eluted with 30 µl RNase-free water and kept at -80°C.

3.5 Detection of 18SrRNA, *pvs16, pvs25 transcripts.*

All RNA samples were determined for *Plasmodium* infection by QMAL and RT-QMAL assay, a genus specific probe base qPCR and RT-qPCR targeting a conserved region in housekeeping *18s rRNA* gene of plasmodium [25]. To ensure gDNA elimination, each sample was tested by QMAL targeting the *18s rRNA* gene in an independent reaction. RT-QMAL or one step RT-qPCR was performed in all RNA samples by using QMAL primers and probe to quantify RNA level in each sample. All QMAL used iTaq Universal Probes Supermix (Bio-Rad, USA) and all RT-QMAL used Suprescript III One Step Quantitative RT-PCR System (Invitrogen, Massachusetts, USA). The reaction without RNA was used for negative control. Each reaction was performed in a total volume of 12 and 2 µL of RNA. All specific primers and conditions for QMAL and RT-QMAL were shown in Supplement Table S7-S13.

3.6 Detection of 18SrRNA, *pvs16, pvs25 transcripts*

For quantification of *P. vivax* gametocyte, qRT-PCR of *pvs16* and *pvs25* transcripts were performed. The amplification of pvs25 was perform using primers and probe according to publish protocol [25]. Probe and primers for *pvs16* was developed using the Vector NTI software (Thermo Fisher Scientific, USA). To eliminate the chance of nonspecific amplification, primers and probe were check for their homology in other plasmodium sp and human genome. The 144 bp amplified *pvs16* gene fragment was cloned into the TA cloning vector (RBC Bioscience, Taiwan) and purified as *pvs16* standard plasmid. One step RT-qPCR of 18srRNA, pvs16, and pvs25 were performed in duplicates on all samples. The standard curves were generated from pvs16 and pvs25 plasmids at 10-10^6 copies/reaction. The reaction was run on CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad) and analyzed with CFX Manager Software Version 3.0 (Bio-Rad Laboratories, California, USA). Value are reported as copies per µl of equivalent blood volume

บทที่ 4 ผลการศึกษา และบทสรุป

4.1 Prevalence of erythrocytic stages of *P. vivax* samples

A total of 130 individuals of all *P. vivax* blood samples set; 10 *P. vivax* participants with individual 13 points of two folds serial dilutions, were conducted in this study. The distribution of *P. vivax* infection as determined by light microscopic; parasite density (0.03-1,327 parasites/µL blood), gametocyte density (0-341 gametocytes/µL blood), female gametocyte density (0-317 female gametocytes/µL blood), male gametocyte density (0-47 male gametocytes/µL blood), was presented in Additional file Table S16-S25.

4.2 Mosquito infection

Oocysts prevalence (mosquito infection rate) was calculated from number of positive mosquitoes with oocysts in total dissected mosquitoes. *An. dirus* mosquitoes fed with each *P. vivax* blood sample developed an infection rate from 0 and 100%, with oocyst loads ranging between 1 and 258.5 per midgut. Mosquito infection rate and oocyst density from individual *P. vivax* infected sample were shown in Additional file Table S16-S25.

4.3 *P. vivax* and mosquito infection

The lowest of parasite density that can infect mosquito was 417 parasites/mL blood. However, median value of the lowest parasite density that can infect mosquitoes from all *P. vivax* infected blood was 5,045 parasites/mL blood. Parallel with parasite density, relation of gametocyte density and mosquito infection was performed, this study showed the lowest gametocyte density that could infect mosquito was 26 gametocytes /mL blood. Median value at lowest gametocyte density that could infect mosquitoes was 223 gametocytes /mL blood. Details of the first mosquito infection of each sample were presented in Table 1. The relative correlations between parasite density (gametocyte density) and mosquito infection rate were shown (Figure 1A and 1B). Moreover, MFA also presented the positive correlation between parasite density (gametocyte density) and mean oocysts per gut (Figure 1C and 1D). In this population group, there were positive correlation between parasite density and both of female and male gametocyte ($p<0.0001$) (Figure 2A). Proportion of female gametocyte correlated with male gametocyte as $R^2= 0.6577$, $p<0.0001$(Figure 2B).

Table 1 The lowest parasite and gametocyte density of each sample for mosquito infection

Case ID	Lowest Mosquito infection rate (%)	Lowest Parasite Density (Parasites/mL blood)	Lowest Gametocyte density (Gametocytes/mL blood)
VTTY121	1.3	2,000	562
VTTY104	33.3	25,312	125
VUBR24	6.5	417	26
VUBR28	1.8	41,469	10,656
VUBR33	1.7	2,051	133
VUBR25	2.9	805	63
VUNL24	1.4	23,844	2,750
VUNL25	5.1	16,000	313
VUNL27	3.2	8,039	1,398
VUNL28	4.1	434	43
Median		5,045	223

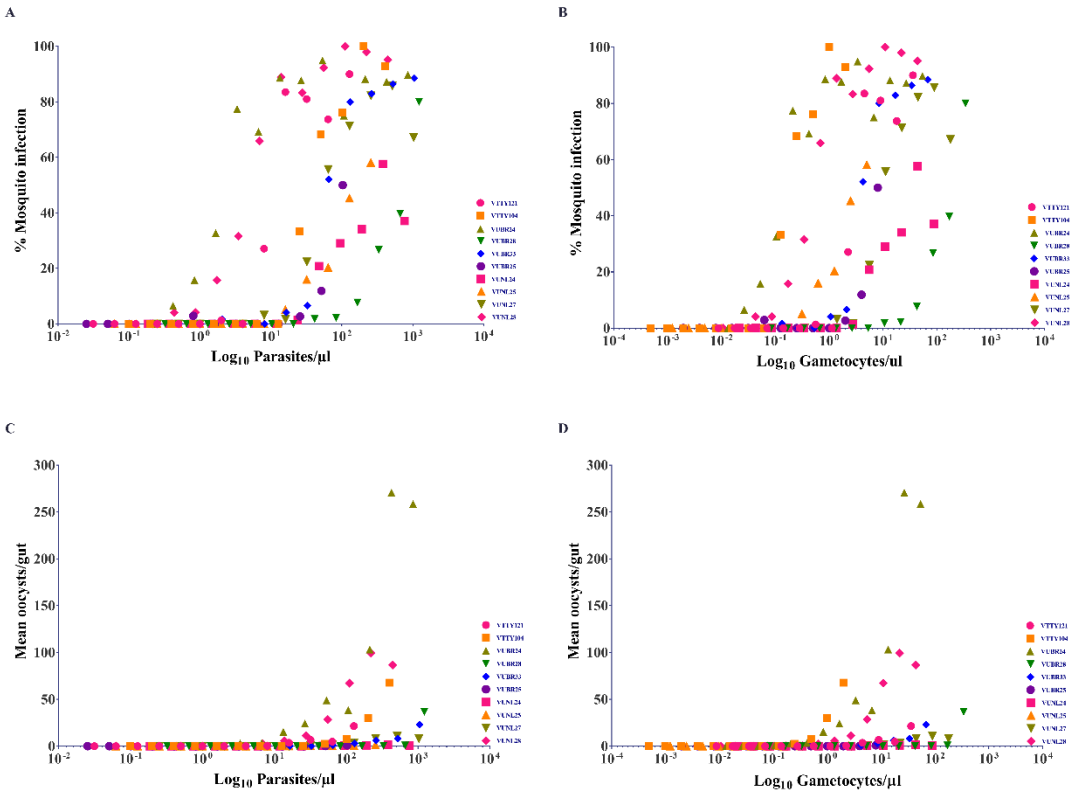

Fig. 1. The correlation of parasite density and mosquito infection rate (A) gametocyte density and mosquito infection rate (B) parasite density and mean oocysts per gut (C) and gametocyte density and mean oocysts per gut (D). Each color represents the percentage of mosquito infection of individual isolate.

4.4 Molecular detection of gametocyte

4.4.1 *Pvs25* and *Pvs16* detection

The expression level of stage specific gene in gametocyte, *Pvs16* and *Pvs25*, were detected by qRT-PCR. RNA from all *P. vivax* infected blood sample were extracted and used as temple for detection. *18s rRNA* was detected as control for positive *Plasmodium* genus by qPCR. There were positive correlation between gametocyte density and expression level of *Pvs16* (R^2= 0.4125, p<0.0001) and *Pvs25* (R^2= 0.7069, p<0.0001) (Figure 2C and 2D).

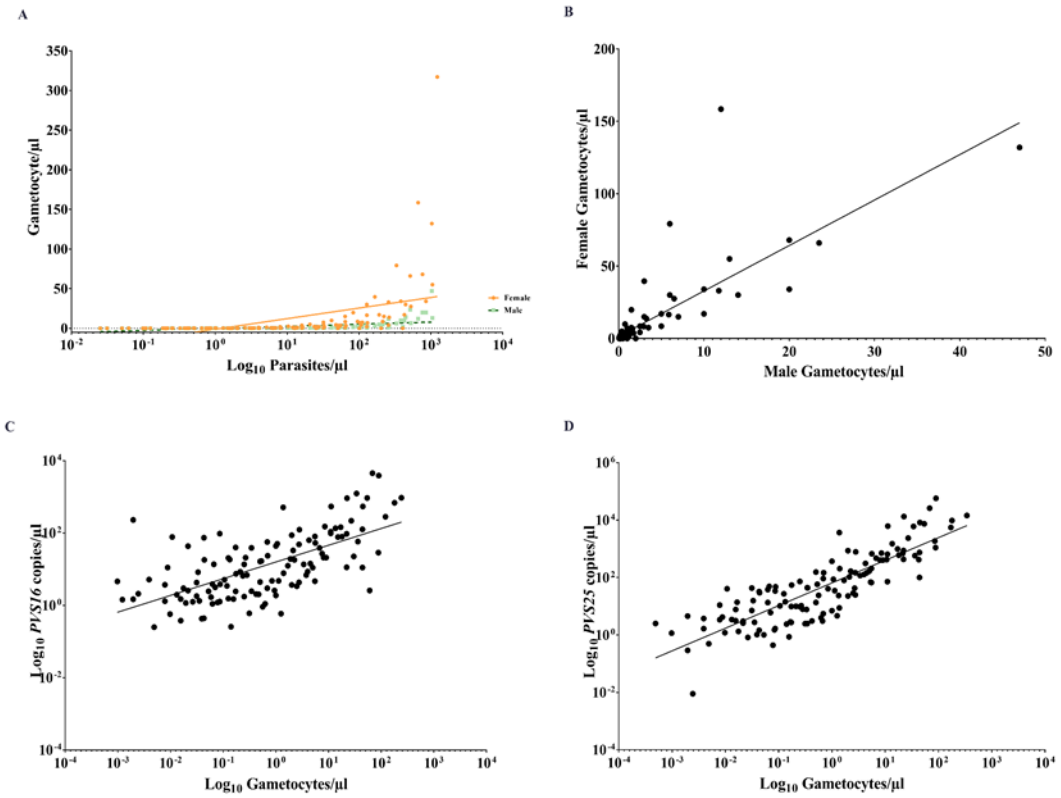

Fig. 2. The correlation of parasite density and gametocyte density (A), Male gametocyte and female gametocyte (B), gametocyte density and *Pvs16* expression (C) and *Pvs25* expression level (D).

4.4.2 Relationship between *Pvs25, Pvs16* and mosquito infection

In this study, Median of *Pvs25* and *Pvs16* copies number of *P. vivax* that can cause the infection in mosquito (10% mosquito infection rate) was 55 copies no/µL and 8 copies no. /µL, respectively (Figure 3A and 3B). Both *Pvs25* and *Pvs16* can be used for gametocyte detection. However, *Pvs16* shows higher sensitivity compared to *Pvs25* in this study group. There were positive correlation between gene expression and mean oocysts density as well (Figure 3C and 3D).

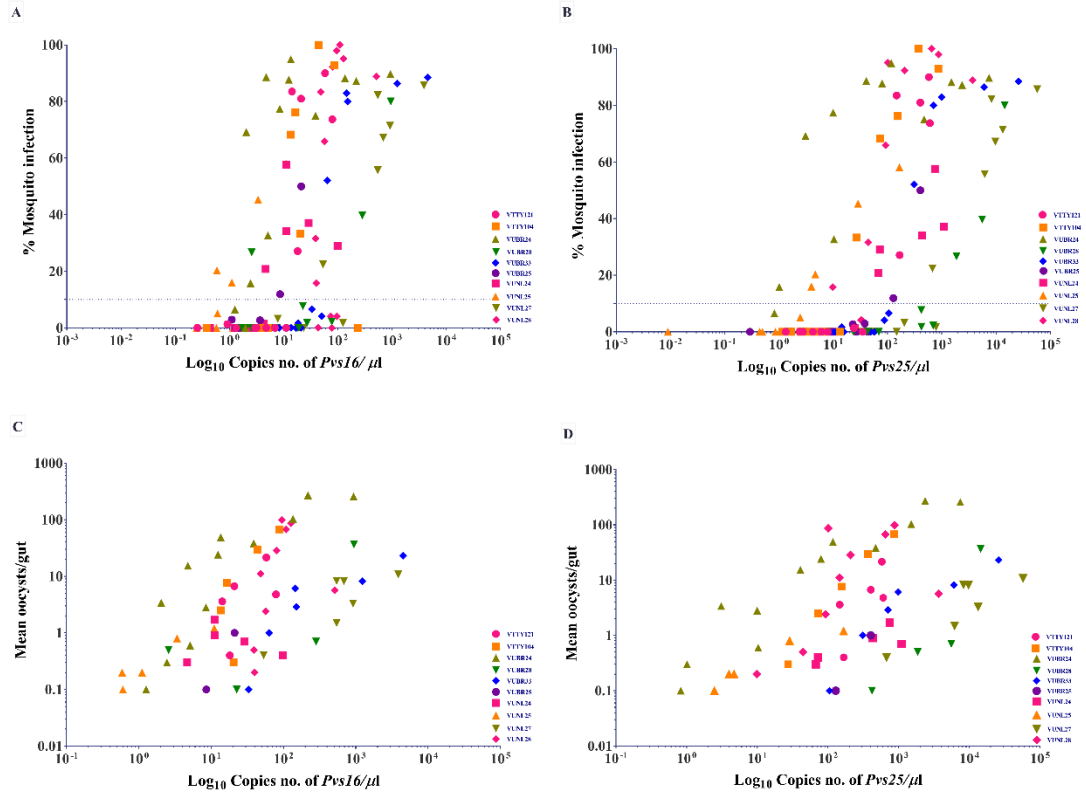

Fig. 3. The correlation of mosquito infection rate and *Pvs16* expression (A) *Pvs25* expression (B). Oocyst density relate with *Pvs16* expression (C) *Pvs25* expression (D). Each color represents the percentage of mosquito infection of individual isolate.

4.4.3 Relationship between light microscope and molecular tools

There are positive correlation between light microscope (parasite and gametocyte density) and molecular techniques detection (*Pvs25* and *Pvs16* detection) in the following relations: *Pvs25* (or *Pvs16*) detection and parasite density, gametocyte density, female gametocyte density and male gametocyte density. There were positive correlations between the level of *Pvs25* ($p<0.0001$, $R^2= 0.6403$), *Pvs16* ($p<0.0001$, $R^2= 0.3848$) and parasite density. Moreover, the positive correlation of both target genes; *Pvs25* ($p<0.0001$, $R^2= 0.713$), *Pvs16* ($p<0.0001$, $R^2= 0.4125$) and gametocyte density were also presented. The results also presented the positive correlation between female gametocyte and the level of *Pvs25* ($p<0.0001$, $R^2= 0.7103$) and *Pvs16* ($p<0.0001$, $R^2= 0.466$). There was, positive correlation between the expression level of *Pvs25* ($p<0.0001$, $R^2= 0.7420$), *Pvs16* ($p<0.0001$, $R^2= 0.4741$) and male gametocyte.

4.4.4 Relationship between the oocyst density and mosquito infection

The mosquito infection rate was strongly correlated with oocysts density (Figure 4) as following best fit value; LogEC50 = -0.01624, HillSlope = 0.8373, S = 1.893, Top = 95.28 and EC50 = 0.9633, N=120)

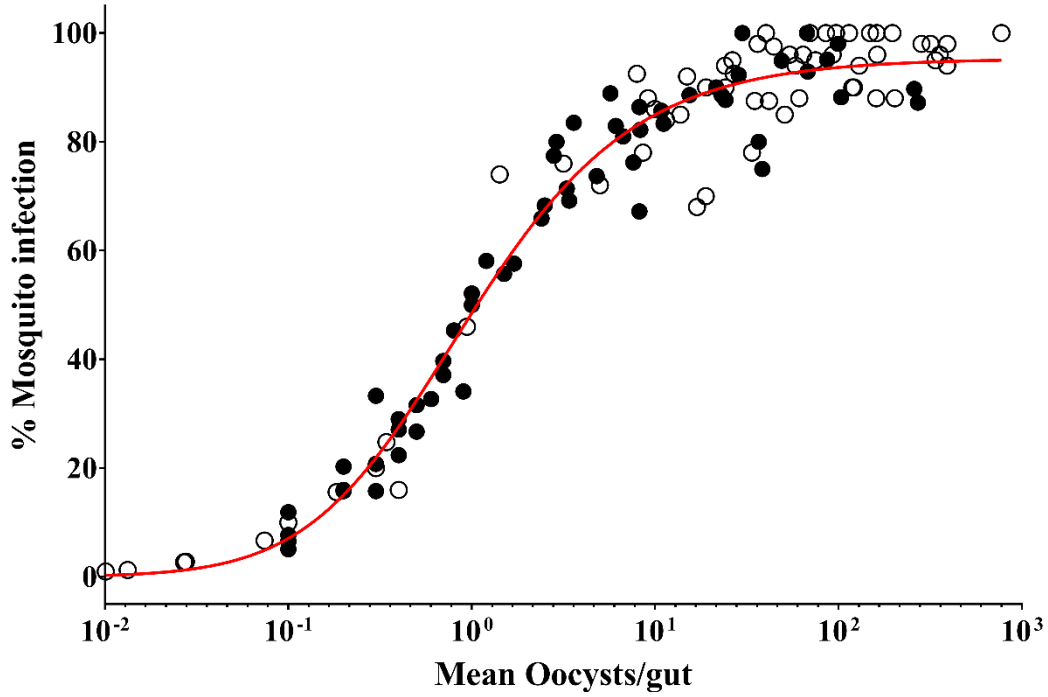

Fig. 4. Relationship between the oocyst density and mosquito infection. Black circles represent values from individual sample in our study. White circles represent values from individual feeding experiments with AB serum replacement. Solid red line is the best fit of the infection process.

Discussion

Thailand is a malaria endemic country and implementing the malaria elimination. *P. vivax* is one of the predominant species causing malarial infection and disease in Thailand. However, there are very limited information on *P. vivax* transmission from human to mosquito. Because of the majority of *P. vivax* infection is very low density of parasite and asymptomatic, it is very important to evaluate how well *P. vivax* infection transmit. To demonstrates the transmissibility of *P. vivax* at very low (parasite) gametocyte density and develop surrogate molecular markers for detection of infective gametocyte, serial dilution of infected *P. vivax* blood from patients was performed to this study as a presenter of very low (parasite) gametocyte density or asymptomatic carrier in endemic areas. In Thailand, the incidence of malaria has been reduced as a results of Thai malaria control programs in clinical case management and vector control. The highest incidence of malaria transmission was mostly located in national borders with Myanmar and Cambodia. In this study, all of *P. vivax* participant's samples were collected from study sites located next to Myanmar border; Tha Song Yang District of Tak Province, and Cambodia and Laos border; Buntarik District and Na Chaluai District of Ubonrajthani Province. Both sites were located on the hilly terrain and populations were occupied primarily by agricultural Karen and Thai residents. The previous cross-sectional malaria surveys conducted in north-western Thailand and Cambodia also indicated the *P. vivax* distribution in theses population [26-27]. Although gametocyte presences in human blood is crucial for an efficient transmission, it was rarely detected by light microscope [28]. With the alternative method; sensitive molecular method, it is now clear that gametocyte is present in most malaria infections and at a highly variable densities that can successfully infect mosquito [29]. All serial dilutions of *P. vivax* infected blood in this study were used as a presenter of sub-microscopic carrier in endemic area to contribute the knowledge of malaria transmission. Mosquito feeding assay (MFA) has been routinely used to quantify the infectiousness of individual. In this study, MFA was performed as a method for mosquito feeding. Although direct skin feeding is more accurate estimate of human transmission potential, MFA is more appropriate to compare infectiousness between individuals and to assess transmission study. Because of direct skin feeding could expose subjects to malaria infection if the mosquito are not reared under sterile conditions. This study showed the positive correlation between parasite, gametocyte density and mosquito infection rate. There were several publications showed the level of parasite density was strongly correlated with gametocyte density and the probability of mosquito infection [30-31]. There are several reports of infectivity of mosquito by undetected *P. vivax* gametocyte have been published in *P. vivax* and *P. falciparum* [32-34]. Comparison study of infectivity between *P. falciparum* and *P. vivax*, they found that malaria transmission by *P. vivax* is likely to be highly efficient with low gametocyte density [35]. The transmission success may depend on the duration of infection including level of gametocyte maturation and proportion of sex ratio gametocyte [36].This study reported positive correlation of sex ratio gametocyte and mosquito infection rate. Sex ratio of gametocyte is one of the critical factors of malaria transmission. Because of one male gametocyte produces eight microgametes during final maturation, whereas one female gametocyte develops into one macrogamete. Therefore, malaria parasites generally produce more female than male gametocyte. An optimal ratio of three or four females to one male gametocyte is commonly observed in *P. falciparum* [37] and two females to one male in *P. vivax*[38]. A higher production of male gametocyte

maximizes the success of transmission and this is especially important at lower gametocyte density. However, the presence of different parasites clones will favor shifting the female-biased sex ratio to enhance the chance of transmission of an individual parasites clones. The important report indicated using light microscope to quantify sex gametocyte which typically observe less than 10 gametocytes in blood smear, not all of them can be reliable number count [28]. These small numbers limit the precision of sex ratio estimates. The molecular assay is very useful to detect gametocyte specific mRNA marker and useful to evaluate the association between *P. vivax* gametocyte density and mosquito infection. Molecular gametocyte detection tools are based on the amplification of RNA that is expressed exclusively in gametocyte and offer possibilities for more detailed characterization of sub-microscopic of *P. vivax* [39]. RNA is required specifically for gametocyte, as asexual parasites also carry the DNA encoding gametocyte specific RNA transcript. For molecular tool detection in this study, determining the transmissibility of *P. vivax* was performed by qRT-PCR. Two sexual stage specific molecular markers; *Pvs16* [40] and *Pvs25* [41] which are *P. falciparum* orthologues known to define all stages and stage V (mature) female gametocyte respectively, were used to quantify copy number of both genes as a measure for gametocyte abundance. The transcripts copy number of each gene was converted into gametocyte density. There are some publications were reported the infectivity of low or undetectable gametocyte density. Kiattibutr *et al.* reported the infectivity of *P. vivax* in low or undetectable gametocyte density from 107 blood samples while Tadesse *et at.* reported from 42 blood samples. In this study, serial dilution of *P. vivax* infected blood sample was performed and presented the same data as previous studies. Thus, the method of serial dilution of blood sample can use as a presenter of very low or undetectable gametocyte to contribute the knowledge of malaria transmission. The infectivity of undetectable or low gametocyte density has been reported by numerous studies [42]. In this study, qRT-PCR showed the lowest parasite infect mosquito at 26 copies /µL blood (or 55 copies /µL blood at median of 10% mosquito infection) by *Pvs25* detection. Moreover, 6 copies /µL blood was detected by *Pvs16* at the lowest parasite infect mosquito (or 8 copies /µL blood at median of 10% mosquito infection). This results agreed with previous publications that presented the range of sensitivity in 0.02 to 0.1 gametocytes/µL or perhaps as low as 0.002 gametocytes/µL by molecular detection tools [28]. In reality, the sensitivity is limited by the volume of blood that can be collected and processed ethically and practically. However, recently publication used high volume *18s rRNA* to determine the distribution of parasites density in endemic area of Thailand (43). The correlation between parasite detection by light microscope and molecular tool; *Pvs25* and *Pvs16* detection, was conducted. The results showed positive correlation of parasite density and both gene; *Pvs25* and *Pvs16,* expression. Furthermore, the correlation between gametocyte detection by light microscope and molecular tool; *Pvs25* and *Pvs16* detection, was also performed. The results showed positive correlation of gametocyte density and both gene; *Pvs25* and *Pvs16,* expression. Previous publication indicated that based on molecular assay, *Pvs25* mRNA reported 8 folds increased gametocyte detection rate compared to light microscope [44]. Although previous reports demonstrated *Pvs25* were detected as early as day 7 after infection and the rate of *Pvs16* expression was 50 times higher than *Pvs25* [45], but this study found higher of *Pvs25* expression than *Pvs16* at day 7 after mosquito feeding. This can be explained that at the time of blood collection from individual participant, most of gametocyte in blood already developed into mature gametocyte. In other study in asymptomatic individual, the mass of circulating parasites is mostly gametocyte. The expression of *Pvs25* in asymptomatic was almost 2 folds higher than early infected participants, even parasitemia was 20 folds lower[46]. Thus, the successful infection of mosquito with

blood from sub-microscopic carries emphasize the importance of targeting all parasites carriers as they should be considered potential malaria transmitters. In this study, both *Pvs25* and *Pvs16* were successful performed as molecular marker for gametocyte detection in sub-microscopic carrier. *Pvs25* and *Pvs16* can use for further study in malaria transmission as a marker for gametocyte detection.

บทที่ 5 ผลลัพธ์และประโยชน์ที่ได้จากงานวิจัย

This study shows the potential of very low *P. vivax* gametocyte density in malaria transmission from human to mosquito. Using molecular markers (pvs16 and pvs25) in identifying gametocyte carriers especially in the asymptomatic individuals provide the advantages over routine microscopic diagnosis as it is way more sensitive. As the gametocytogenesis of *P. vivax* occurs at much earlier before the onset of the symptom which allow the parasites to be transmitted. The early diagnosis especially for the asymptomatic carriers would resulting in the early disruption of the transmission. Therefore, these results indicated that the molecular detection of the parasites/gametocytes could help strengthen the malaria control program and should be considered for new policies for malaria control strategies targeting sub-microscopic carriers as a tool for malaria elimination.

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ภาคผนวก

Supplementary data

Table S1 The sequence of primer for primary nested PCR

Name	5'-3'
P1F	ACG ATC AGA TAC CGT CGT AAT CTT
P2R	GAA CCC AAA GAC TTT GAT TTC TCA T

Table S2 The sequence of reverse primer for secondary nested PCR

Name	Species	5'-3'
FR	*P. falciparum*	CAA TCT AAA AGT CAC CTC GAA AGA TG
MR_W2	*P. malariae*	AAG GAA GCT ATC TAA AAG AAA CAC TCA
OR	*P. ovale*	ACT GAA GGA AGC AAT CTA AGA AAT TT
VR	*P. vivax*	CAA TCT AAG AAT AAA CTC CGA GAG GAA A
KR_W1	*P. knowlesi*	CTG AAG GAA GCA ATC TAA GAG TTC

Table S3 The composition of nested PCR reaction for first strand

Component	Volume/25 µL reaction	Final concentration
Sterile-MilliQ water	8.5 µL	
2X GoTaqGreen mastermix	12.5 µL	1X
Primer-F	1 µL	0.4 µM
Primer-R	1 µL	0.4 µM
Template DNA	2 µL	0.5 ng

Table S4 PCR cycling profile of nested PCR reaction for first strand

Cycle step	Temperature	Time	Cycles
Initial denaturation	94 °C	10 min	1
Denaturation	95 °C	30 sec	35
Annealing	60 °C	90 sec	
Extension	72 °C	1 min	
Final extension	72 °C	5 min	1
	10 °C	hold	

Table S5 The composition of nested PCR reaction for second amplification

Component	Volume/ 25 µL reaction	Final concentration
Sterile-MilliQ water	9.5 µL	
2X GoTaqGreen mastermix	12.5 µL	1X
Primer P1F	1 µL	0.4 µM
Specific primer	1 µL	0.4 µM
Template DNA (1:50 dilution of the first PCR product were as the template)	1 µL	

Table S6 PCR cycling profile of nested PCR reaction for second amplification

Cycle step	Temperature	Time	Cycles
Initial denaturation	94 °C	10 min	1
Denaturation	95 °C	30 sec	20
Annealing	60 °C	90 sec	
Extension	72 °C	1 min	
Final extension	72 °C	5 min	1
	10 °C	hold	

Table S7 Sequence of primer and probe of qPCR for *18s rRNA* detection

Name	5'-3'
Probe	FAM-TCA ATT CTT TTA ACT TTC TCG CTT GCG CGA –BHQ
Primer F	TTA GAT TGC TTC CTT CAG TRC CTT ATG
Primer R	TGT TGA GTC AAA TTA AGC CGC AA

Table S8 The composition of qPCR reaction for *18s rRNA* detection

Component	Volume/ 12 µL reaction	Final concentration
iTaq™ Universal Probes Supermix	6 µL	1X
Primer F	0.82 µL	0.4 µM
Primer R	0.82 µL	0.4 µM
Probe	0.36 µL	0.4 µM
Template RNA	4 µL	-

Table S9 qPCR cycling profile for *18s rRNA* detection

Cycle step	Temperature	Time	Cycles
Stage1	50°C	15 min	1
Stage2	95 °C	2 min	1
Stage3	95 °C	15 sec	45
	59 °C	20 sec	
	72 °C	30 sec	

Table S10 Sequences of *Pvs25* primer and probe

Name	5'-3'
Pvs25_F	ACA CTT GTG TGC TTG ATG TAT GTC
Pvs25_R	ACT TTG CCA ATA GCA CAT GAG CAA
Pvs25_Probe	FAM –TGC ATT GTT GAG TAC CTC TCG GAA- BHQ1

Table S11 Sequences for *Pvs16* primer and probe

Name	5'-3'
Pvs16_F	ACGTAGCTTCTAAGCGAAGCAA
Pvs16_R	TTAACCTGAACGATTGCCAGATT
Pvs16_Probe	HEX-CAGACGAAGAACACCTCGATCTAAAGTGCA-BHQ1

Table S12 The composition of PCR reaction for *Pvs25, Pvs16* detection

Component	Volume/ 12 µL reaction	Final concentration
SuperScript™III One-Step RT-PCR System with Platinum™Taq DNA Polymerase	6 µL	1X
Primer F	0.82 µL	0.4 µM
Primer R	0.82 µL	0.4 µM
Probe	0.36 µL	0.4 µM
Template RNA	4 µL	-

Table S13 PCR cycling profile for *Pvs25* detection

Cycle step	Temperature	Time	Cycles
Stage1	50°C	15 min	1
Stage2	95 °C	2 min	1
Stage3	95 °C	15 sec	45
	57.2 °C	30 sec	
	60 °C	30 sec	

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PUBLICATIONS

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