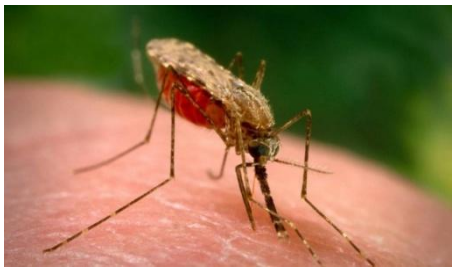
Graduate Programme

Molecular Biology & Biomedicine

Master Thesis

New rapid one-step PCR diagnostic assay for *Plasmodium falciparum* infective mosquitoes



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Abstract

An essential component of malaria vector control programmes is the detection of *Plasmodium falciparum* within its mosquito hosts, particularly in the salivary gland where the infective sporozoites reside. Several protocols have been developed for this purpose; however they require dissection of mosquito specimens prior to analysis, in order to detect the parasite in the salivary gland. Here, a novel one step RT qPCR TaqMan diagnostic assay was developed specifically for mosquitoes with infective *Plasmodium falciparum* sporozoites in the salivary glands. The assay is based on detection of the sporozoite-specific Pf*slarp* and Pf*plp1* gene transcripts. These two transcripts were chosen based on bioinformatics analysis and experimentally verified to be specifically expressed in the salivary gland sporozoite stage of the parasite but not in other mosquito parasite stages. The proof of principle and the performance of the assay were demonstrated using RNA later preserved mosquito samples. Tests of analytical sensitivity showed the novel TaqMan assay to be 100% accurate, although its performance in the field needs to be further demonstrated. This method has no requirement for head/thorax dissection and post-PCR processing and thus is simpler and more rapid to perform in individual mosquitoes or mosquito pools. Importantly it can be used in single or multiplex formats also targeting additional markers expressed in different tissues, such as detoxification enzymes associated with insecticide resistance.

Introduction

Malaria is considered to be one of the most severe infectious diseases worldwide, causing about half a million deaths every year, primarily in the developing world(2016).It is transmitted by *Plasmodium*-infected female *Anophelinae* mosquitoes. Of the five *Plasmodium* species that infect humans *Plasmodium falciparum* is the most important (Miller, Ackerman et al. 2013; White, Pukrittayakamee et al. 2014).The life cycle of *Plasmodium* differentiates between vertebrate hosts and invertebrate vectors. When a female *Plasmodium*-infected mosquito takes a blood meal, parasites (sporozoites) are transmitted to the vertebrate host. Sporozoites enter the blood stream, infect hepatocytes and mature into schizonts which contain thousands of merozoites. Merozoites are invasive forms that invade erythrocytes, where they mature from ring-forms, to trophozoites and finally, to schizonts, containing daughter merozoites, which invade new erythrocytes. This is the asexual blood cycle. The sexual forms of the parasites (male and female gametocytes) are formed within erythrocytes. These sexual stages are ingested by a mosquito during a blood meal, undergo sexual fertilization once and this takes place in the mosquito vector, resulting into zygotes. Motile zygotes are called ookinetes and have the ability to traverse the midgut epithelium. After that they transform into oocysts underneath the extracellular matrix and during the next 10 days sporozoites are formed. When mature these are released into the open hemolymph circulation. The circulating sporozoites recognize mosquito salivary glands, through protein interactions, invade them and are stored there until an infective bite, during which sporozoites are transmitted to a vertebrate host (Whitten, Shiao et al. 2006; Ghosh and Jacobs-Lorena 2009; de Koning-Ward, Gilson et al. 2015)(Figure 1).

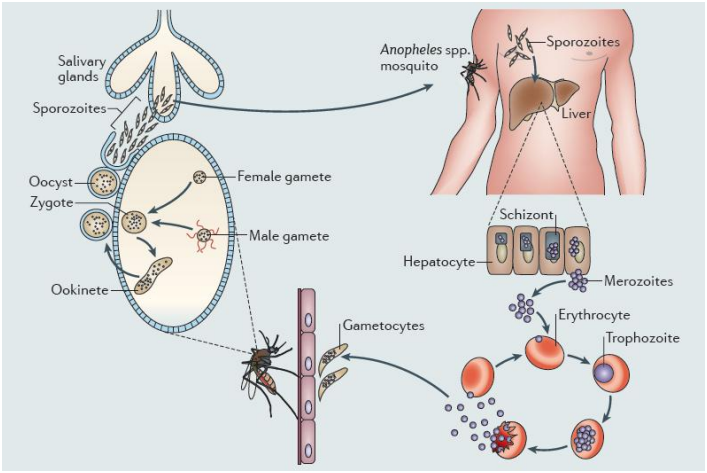

Figure 1. Plasmodium life cycle into the vertebrate and invertebrate host.

(de Koning-Ward,Gilson & Crabb, 2015*)*

Two mosquito epithelial interfaces, of the midgut and salivary glands, interact with parasites at their most vulnerable form as the parasites have to pass through the epithelium of these organs(Whitten, Shiao et al. 2006) . During epithelial traversal the parasites suffer severe losses, as they become exposed to host defense mechanisms. These stages are therefore bottlenecks of the *Plasmodium* life cycle (Whitten, Shiao et al. 2006; Aly, Vaughan et al. 2009). Hence, not all gametocytes ingested by *Anophelinae* mosquitoes result in sporozoites formation in the salivary glands. The invertebrate hosts are considered infectious once the sporozoites have invaded the salivary glands (Bousema, Dinglasan et al. 2012). As mentioned above, infection by *Plasmodium* species causes malaria, the most important arthropod-transmitted infectious disease(Matuschewski 2006). The problem is detected mainly in the developing world, representing the majority of the global population. According to WHO and the most recent World Malaria Report (2016), malaria caused approximately half a million deaths in 2015, 303.000 of which occurred in under-5-years-old children. This makes malaria a major killer, taking the life of one child every two minutes in Sub-Saharian region. (WHO 2016)

During the last years there is a great progress in reducing the deaths caused by this vector-borne disease. Insecticide-treated nets (ITNs), indoor residual spraying (IRS), chemoprevention in pregnant women and children, artemisinin-based combination treatment (ACT), as well as human and vector diagnostic tests contributed to a great extent to the reduction of malaria in the last two decades(Bhatt, Weiss et al. 2015). However, the elimination of the threat from the countries where malaria is transmitted remains a challenge.

Prevention of malaria is best achieved by vector control, which today in Africa relies on the use of insecticides. Monitoring mosquito vector populations is an integral and essential component of most vector control programmes. Contemporary data on mosquito species composition, resistance to insecticides, and actual infection status of mosquito-vectors, are a prerequisite for effective interventions (Vontas, Mitsakakis et al. 2016). The infection status probably represents the most important information, given the number of tests performed in the frame of country level monitoring activities. Thus, the characterization of the infection status of the vectors, in other words the presence of parasites in the salivary glands, is an important parameter that should be taken into account in field settings for efficient control of vector populations(Marie, Boissiere et al. 2013).

To date, the methods used for the scoring of vectors being infectious are either labour intensive, or they have sensitivity and specificity issues. Methods to overcome these difficulties are expensive and thus, not affordable in poor countries.(Marie, Boissiere et al. 2013) Dissection of salivary glands followed by microscopic examinations, or ELISA using an antibody against the Circumsporozoite Protein (CSP) present on sporozoites, has been used extensively for the identification of the epidemiologically relevant sporozoite infected mosquitoes(Wirtz, Zavala et al. 1987; Fontenille, Meunier et al. 2001; Smith, Battle et al. 2012). However, these assays require experienced personnel, particularly for mosquito dissections, while they also have sensitivity and specificity issues, due to hemolymph stage parasite contamination in the specimens that are examined, and often result in overestimation of mosquito infection rates. Research efforts in recent decades were focused on developing diagnostic tools that are based on PCR for detecting Plasmodium falciparum in human blood and in mosquito samples.(Zaman, Tan et al. 2001; Fabre, Berry et al. 2004) Among these Padley et al used a multiplec PCR for detection of the four major Plasmodium species (P.falciparum, P. malariae, P. ovale, P. vivax) in human blood samples.(Padley, Moody et al. 2003) Small SubUnit of ribosomal RNA (SSU Rrna) of Plasmodium species has also been targeted for multiplex PCR assays, but this approach has sensitivity issues, as it requires

significant amount of parasite DNA (Marie, Boissiere et al. 2013). Vernick et al, used reverse transctipase PCR, for detection of this subunit of the sporogonic stages. (Vernick, Keister et al. 1996) For increased sensitivity and specificity quantitative PCR (qPCR) have also been developed to measure infectivity of Plasmodium both in mosquito and in human blood samples. Recently, a duplex real time PCR with primer for SSU rRNA was generated for detection of four Plasmodium species in field mosquitoes from Benin, but even after head-thorax dissection this method gives false positive results for mosquito infection status, as the latter should be based only in sporozoites that have invaded the salivary glands (Sandeu, Moussiliou et al. 2012). In addition, for the measurement of the infective mosquitoes there is a qPCR assay based on the mitochondrial Cytochrome c oxydase subunit 1(COX-1) gene. This method, described by Boissiere et al, is sensitive and specific but requires also throax-head dissection (Boissiere, Gimonneau et al. 2013) (Marie, Boissiere et al. 2013).Moreover t nested PCR approaches(Snounou and Singh 2002), as well as TaqMan diagnostic DNA-based assays are used, which require no post-PCR processing and can discriminate *P. falciparum* from *P. vivax, P. ovale* and *P. malariae(Bass, Nikou et al. 2010)*. However, the latter method is based on DNA detection that also requires the dissection of the mosquito head and thorax and the removal of the abdomen prior to DNA extraction, in order to detect infective mosquitoes(Bass, Nikou et al. 2010). This limits its practicality, particularly when large pools of mosquito samples have to be tested, which is often necessary in low transmission settings.

In this study we identified salivary gland sporozoite specific *Plasmodium* transcripts, which were subsequently used for the development of a specific diagnostic test for detecting mosquitoes carrying infective *P. falciparum* in their salivary glands For the identification of the infectious status of vectors at least one specific marker should be used. This marker should detect the prevalence of the sporozoites in the mosquito salivary glands and should be specific. It is of high importance that a marker gene should not be expressed in midgut sporozoites (oocysts) or in sporozoites that are released in the hemolymph to avoid false positive results (Figure 2).

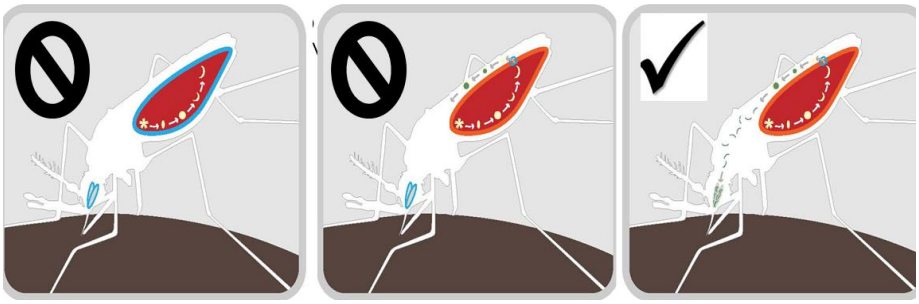

Figure 2 The marker should be specific for the infective stage of sporozoites in the mosquito-vector, that is it should only detect sporozoites after salivary gland invasion (third panel) but not oocysts sporozoites (first panel), nor circulating sporozoites after oocysts rupture (second panel).

Materials and Methods

Ethics Statement

All work was carried out in full conformity with Greek regulations consisting of the Presidential Decree (160/91) and law (2015/92) which implement the directive 86/609/EEC from the European Union and the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes and the new legislation Presidential Decree 56/2013. The experiments were carried out in a certified animal facility license (EL91-BIOexp-02) and the protocol has been approved by the FORTH Committee for Evaluation of Animal Procedures (6740/ 8/10/2014) and by the Prefecture of Crete (license number # 27290, 15/12/2014).This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Animal Care and Use Committee of the Johns Hopkins University (permit number MO15H144). Commercial anonymous human blood was used for parasite cultures and mosquito feeding, and informed consent was therefore not applicable. The Johns Hopkins School of Public Health Ethics Committee has approved this protocol.

Plasmodium berghei life cycle

Anopheles gambiae G3 strain mosquitoes were raised at 28°C, 80% humidity under 12-h light/12h dark cycle and maintained on a 10% sucrose solution during adult stages. 5-6-day old female mosquitoes were blood-fed on anesthetized adult *Mus musculus* mice, that had been infected with *Plasmodium berghei*, strain ANKA 2.34. Rodents were assayed for high levels of parasitemia and the abundance of gametocyte-stage parasites capable of exflagellation. After the infective blood meal, mosquitoes were maintained at 19°C. On day 11 post feeding mosquitoes which had well developed ovaries were dissected in PBS and midguts were isolated and stored at -80°C. Salivary glands were isolated on day 21 post blood-meal.

RNA isolation from different stages of *Plasmodium berghei* and cDNA synthesis

RNA form the dissected midguts and salivary glands was isolated using Trizol reagent protocol (Ambion) following manufacturer's instructions. DNase-treatment followed by purification using Qiagen RNeasy MiniElute Cleanup Kit was done. For cDNA synthesis 1μg of RNA was reverse transcribed using Thermoscirpt RT-PCR system kit (Invitrogen), with oligonucleotide primers and following manufacturer's instruction. To confirm that cDNA was successfully synthesized, PCR using primer pairs specific for RPS7 (*A. gambiae* Ribosomal Protein 7) was caried out.

Plasmodium falciparum infected mosquitoes

Anopheles gambiae strain KEELE strain mosquitoes were fed on a NF54 (MR4) *P. falciparum* gametocyte culture through artificial membranes at 37^{o}C. The median number of oocysts/midgut was 2(range of 0-42 oocysts/midgut) for a subset of mosquitoes (N=22) on day 7 post gametocyte ingestion. Mosquitoes were collected at 12 and 21 days in RNAlater and shipped at ambient temperature.

RNA isolation from different stages of *Plasmodium falciparum* and cDNA synthesis

RNA from whole mosquitoes was isolated using PicoPure kit (Arcturus) according to the manufacturer's instructions. After the extraction DNase treatment and cDNA synthesis were carried out as described above.

Comparison of expression levels between midgut and salivary gland sprorozoites in *Plasmodium berghei* and *Plasmodium falciparum*

Initially primer pairs for every gene were designed and PCR conditions were optimized using gradient PCR (Table 1 and 2). Polymerase chain reaction (PCR) using GoTaq Pro polymerase (Promega) was carried out as described by the manufacturers in a BioRad thermal cycler. For testing each primer pair a positive control containing genomic parasite DNA as template and a negative control without template were included in each experiment. For the five first *P. berghei* genes tested (Pbs*larp,* Pb*gest,*Pb*plp1,*Pbs*patr,* Pbs*pect1*) 35 PCR cycles were used. For the *P. bergheiuis* genesand for all *P. falciparum* genes tested, samples were removed after 25, 30 and 35 PCR cycles.

Table 1 Primer sequences and main PCR conditions for the thirteen *P. berghei* genes tested

Gene	Forward primer (5´-3´)	Reverse primer (5´-3´)	Amplicon size gDNA(bp)	Amplicon size transcript (bp)
Pb*uis1*	GGATGGGCAAAGACAAAGAA	TCACCTATTGTGCTCCACCA	**726**	**726**
Pb*uis2*	CCCGAGGTAGATGAATTGGA	GCTCACGGAAGGATCGATAA	**622**	**622**
Pb*uis4*	CCCATTGATGAGACAAACGA	ATGCTTCTTCAGTGGGGCTA	**415**	**415**
Pb*uis5*	GGTTGGGAATCAGAACAAGC	TGCTGAACCCGAACTTAGTG	**870**	**870**
Pb*uis10*	TGCAGAGGGTACTGTTGACG	TCGAACCAGCAAATGGTACA	**689**	**689**
Pb*uis12*	TGCAGAAGACAGCGAATTGT	TCTTCCCGTTTTTCCAACAC	**816**	**816**
Pb*uis24*	GGAAGGAAAGCAAGGAAAGG	GGTCCTGTTTGATTGGCAGT	**807**	**807**
Pb*csp*	AGCATCCAAGCCCAAAGG	CCGCGCTTGGGATATAAG	**697**	**697**
Pb*spect1*	AAGCATTGAACCAAAAGGAA	TTTTTGCTTCTCCTTTTCCA	**683**	**455**
Pb*slarp*	TGAACCCAAATGATCAAGCA	GAATCGGCACAAGGCACTAT	**874**	**773**
Pb*gest*	TAATTCCCTCGGGAACAAGC	TTGGTGGCTTCCAATGTTTT	**835**	**480**
Pb*plp1*	CAAGCGTAGGGGGATCTACA	CTTTCCCCGATGAAGATGAA	**898**	**698**
Pb*spatr*	CCTGATGTTGGTGCAGACAC	TCCAATCCGACCAAGGACTA	**793**	**457**

Table 2 Primer sequences and main PCR conditions for the six *P. falciparum* genes tested

Gene	Forward primer (5´-3´)	Reverse primer (5´-3´)	Amplicon size gDNA(bp)	Amplicon size transcript (bp)
Pf*uis4*	ATGAAGGTTTCTAGGCATACCG	GTCGGATCCATCATTCACCT	**278**	**278**
Pf*uis10*	TTTAGCGAGTTTTCGGAGGA	CATCAGCGGGGAAATTCTTA	**865**	**865**
Pf*uis24*	TGTGAATCCTGACGAAGCTG	GGGCTCCTTGTTGAGCATT	**743**	**743**
Pf*csp*	CGGATGGTAATCCTGATCCA	ACATGGGGACCATTCAGTTG	**710**	**710**
Pf*slarp*	GAATGATCTTAATTCCAATGAGC	CAGCCCTGGTATATAAATTACTGTC	**1144**	**938**
Pf*plp1*	AGTGCTGGAGGATCTACTGATG	CATGTCCAGAACCATGAACAG	**1259**	**1027**

Real-time qRT-PCR of *P. falciparum* infected mosquitoes

Total RNA was extracted from mosquito pools 12- and 21- days post infection (N = 5 pools of 10 individuals, from each stage) using TRI Reagent™ Solution (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Total RNA concentration was determined by spectrophotometry using a NanoDrop 2000c spectrophotometer (Thermo Scientific) and its integrity was assessed via agarose gel electrophoresis (1.0% w/v). A quantitative Reverse Transcription-real-time PCR (qRT-PCR) assay, based on TaqMan® chemistry, was designed and developed for the quantification of Pb*slarp* (Accesion no. XM_001348111.1) and Pb*plp1* (XM_001349297.1) target genes, normalized to Pb*csp* reference gene expression (XM_001351086.1). Gene-specific primers and probes were designed using the Primer Express software v 3.01 (Applied Biosystems, Foster City, CA). For the target genes, one primer spanned two exons in order to avoid DNA amplification. Each probe was labelled with a different fluorescent dye in order to evaluate the possibility of multiplexing. The analytical parameters of the qRT-PCR reactions are presented in Table 3. Reactions were performed in the CFX Connect™ Real-Time PCR system (Bio-Rad, Hercules, CA, USA) using a one-step RT-PCR mastermix supplied by FTD (Fast-track diagnostics, Luxembourg) and total RNA of at least 2.0 µg per sample in a total reaction volume

of 10 μL. The thermal cycle parameters were: 50°C for 15 min, 95°C for 3 min, and 40 cycles of 95°C for3 sec and 60°C for 30 sec, allowing a sample to result time of ~75 min. Samples were amplified in triplicates and each run always included a non-template control. *P. falciparum* DNA was also tested and no detectable signal was observed for the target genes. Additionally, both DNase and non-DNase treated aliquots of the same samples were assessed and no difference was observed in normalized expression levels (data not shown). The comparative Ct method was used for the calculation of Relative Quantification (RQ) units for each sample and each target gene ($RQ = 2^{-dCt}$, where dCt = (Ct target - Ct reference). Comparison of expression levels between the two groups was performed with the independent samples t-test (SPSS v17.0).

Table 3 Analytical parameters of the qRT-PCR reactions

Reaction parameters				
Gene	Forward primer(5′-3′) (concentration in nM)	Reverse Primer (5′-3′) (concentration in nM)	Probe (concentration in nM)	Amplicon size(bp)
Pf*slarp*	CCAAACACTCAGCACAGGAACA (500)	CCATACAGCCCTGGTATATAAATTACTG (500)	FAM-ATGTCTATTGGCACTTACT-MGB (300)	131
Pf*plp1*	CCTTTTAGGGTTTGGTATATCCTCTTC (200)	GAGCAGCTTTTCATTCCTGGT (200)	HEX-TCAGGGAGAATCAATTC-MGB (250)	96
Pf*csp*	TCAACTGAATGGTCCCCATGT (100)	GAGCCAGGCTTTATTCTAACTTGAAT (200)	Cy5-TGTAACTTGTGGAAATGG-MGB-(250)	68
Quality control				
Gene	Reaction Efficiency (%)	Linearity (R^2)	Dynamic Range (Ct)	
Pf*slapr*	108.5	0.9992	24.0 -34.0	
Pf*plp1*	99.70	0.9969	26.0-36.0	
Pf*csp*	95.5	0.9996	16.0 - 30.0	
Multiplexing				
3plex: Pf*slarp* + Pf*plp1* +Pf*csp*		2-plex (A): Pf*slarp*+Pf*csp*	2-plex (B): Pf*plp1*+Pf*csp*	
Incompatible		Compatible, but with loss in sensitivity (+1.5 Ct shift for *Pfslarp*)	Compatible	

Results

Identification of salivary gland-specific *Plasmodium berghei* sporozoite transcripts

As an initial test for the identification of a transcriptional marker for salivary gland -stage sporozoites we used the rodent model parasite *P. berghei*. Due to the absence of transcriptomic data for the stages of interest we used proteomic data in Plasmodb (www.Plasmodb.org) to identify candidate genes. Five genes were initially selected: Pb*plp1* (perforin-like protein 1, also called Pb*spect2*, PBANKA_1006300), Pb*gest* (gamete egress and sporozoite traversal protein, PBANKA_1312700), Pb*spatr* (secreted protein with altered thrombospondin repeat domain, PBANKA_0309500), Pb*slarp* (sporozoite and liver-stage asparagin-rich protein, PBANKA_0902100) and Pb*spect1* (sporozoitemicronemal protein essential for cell traversal, PBANKA_1355600). For the preparation of the tissue-specific templates, midguts and salivary glands were isolated from *A. gambiae* mosquitoes, previously fed on *P. berghe*i-infected mice. Midguts were dissected on day 11and salivary glands on day 21 post infectious blood meal. The presence of oocysts (2 to 10 per infected mosquito) was confirmed in mosquito guts in parallel infections. Semi-quantitative reverse transcribed PCR, using specific gene primers, was then carried out. These experiments revealed that the transcript levels of Pb*gest*, Pb*plp1* and Pb*slarp* were higher in salivary gland sporozoites, but they were also detected in the midgut samples at similar levels (Figure3). The discrepancy with the proteomic results suggests that the expression might be regulated post-transcriptionally, although technical/sensitivity issues that may have restricted the proteomic analysis cannot be excluded.

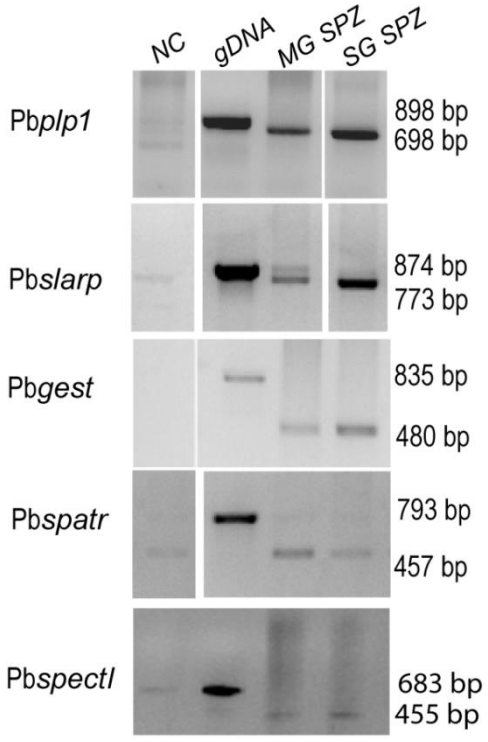

Figure 3. RT-PCR analysis of *P.berghei* Pb*plp1*, Pb*gest*, Pb*spectI*, Pb*spatr and* Pb*slarp* transcripts. Templates were derived from gDNA (lane 2), dissected midguts at 11 d pbm(post blood meal)sporozoites (lane 3, MG SPZ) and dissected salivary glands 21 d pbm (lane 4 SG SPZ). Lane 1 is the negative control without template (NC). The samples were amplified for 35 cycles. Molecular weight of the amplified fragments is indicated to the right.

We also tested expression of the *uis* (up-regulated in infective sporozoites) genes. These genes were identified using a suppression subtractive hybridization method, which revealed the highly up-regulated transcripts in salivary gland sporozoites, as opposed to midgut sporozoites(Matuschewski, Ross et al. 2002). We selected the genes Pb*uis1* (serine-threonine protein kinase, PBANKA_0205800), Pb*uis2* (serine-threonine protein phosphatase, PBANKA_1328000), Pb*uis4* (early transcribed membrane protein, PBANKA_0501200), Pb*uis5* (cysteine desulfatase, PBANKA_0211300), Pbuis24 (heat-shock protein 70, PBANKA_0914400), Pb*uis12* (RNA-binding proten, PBANKA_0506200) and Pb*uis10* (phospholipase, PBANKA_1128100). RNAseq data (www.PlasmoDB.org) indicated that the orthologues of these *P. berghei* genes in *P. falciparum* had high expression levels at the sporozoites stage. We used the Pb*csp* gene transcript as a positive control for the presence of parasites in our samples. The transcript abundance of this gene was similar in our infected *P. berghei* midgut and salivary gland samples. In these experiments we retrieved samples from the PCR reactions at 25, 30 and 35 cycles to avoid saturating the reaction, and to improve quantification.

The results of the RT-PCR analysis of the *uis* gene transcripts showed that Pb*uis4* was highly expressed in the salivary gland-stage, already being detected after 25 PCR cycles. Pb*uis10* and Pb*uis24* showed a more abundant amplification product in salivary gland compared to midgut samples, but the difference was modest (Figure 4).The other tested genes were either not expressed in at the salivary gland stage, or the difference between midgut and salivary gland samples were negligible (Figure 5).

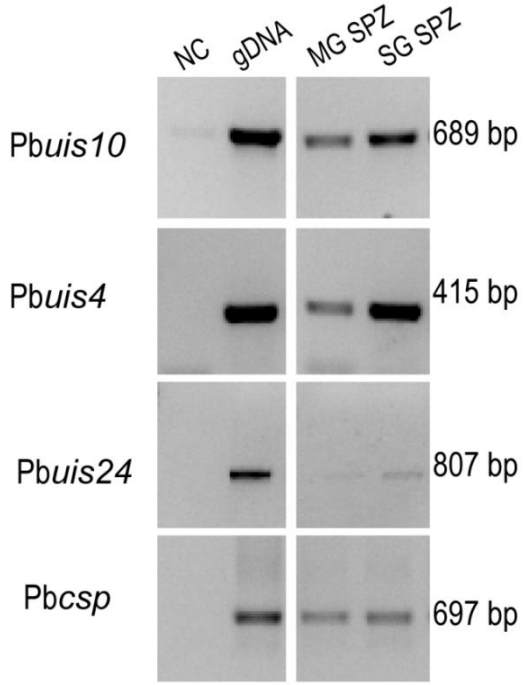

Figure 4. RT-PCR analysis of *P.berghei* Pb*uis4*, Pb*uis10*, Pb*uis24* and Pb*csp* transcripts. Templates were derived from gDNA (lane 2), dissected midguts 11 d pbm sporozoites (lane 3, MG SPZ) and from dissected salivary glands 21 d pbm (lane 4 SG SPZ). Lane 1 is the negative control without template (NC). The samples were amplified for 35 cycles. Molecular weight of the amplified fragments is indicated to the right.

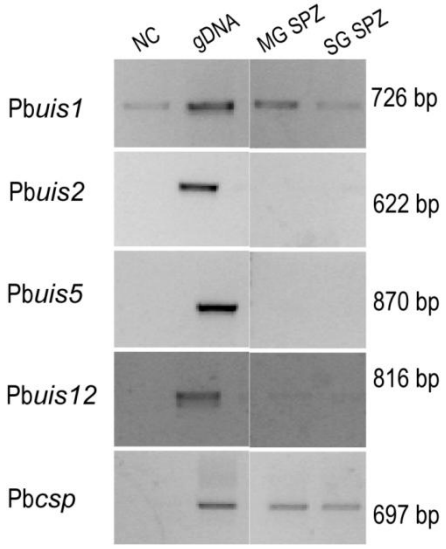

Figure 5. RT-PCR analysis of *P.berghei* Pb*uis1*, Pb*uis2*, Pb*uis5* and Pb*uis12* transcripts. Templates were derived from gDNA (lane 2), dissected midguts at 11 d pbm sporozoites (lane 3, MG SPZ) and dissected salivary glands at 21 d pbm (lane 4 SG SPZ). Lane 1 is the negative control without added template (NC). The samples were amplified for 35 cycles. Molecular weight of the amplified fragments is indicated to the right.

Identification of *Plasmodium falciparum* salivary gland-specific transcripts

The above results suggested that it is possible to use RT-PCR to specifically identify mosquitoes carrying parasites in their salivary gland. To determine whether this is also applied to the human parasite *P. falciparum* we performed a similar analysis on whole infected mosquito samples that had been stored 12 or 21 days prior to dissection. Samples from the first time point contain only midgut sporozoites in oocyst, while in the second time point samples contain salivary gland -invaded sporozoites. The presence of oocysts and sporozoites was confirmed by microscopic examinations of the same infected mosquito cohorts. Samples containing pools of 10 mosquitoes each were processed for RNA extraction and cDNA synthesis. Based on the *P. berghei* experiments, and *P. falciparum* sequence (Plasmodb), primers were designed and optimized for Pf*slarp*, Pf*plp1*, Pf*uis4*, Pf*uis10* and Pf*uis24* and the Pf*csp* gene was used as a positive control for the presence of parasite RNA in our samples (Table 2). RT-PCR experiments were carried out and amplification products were analysed after different number of cycles (30, 35 and40). The results showed that Pf*uis10* was not amplified from either stage and the Pf*uis24* gene transcript was amplified at similar levels in both samples (Figure 6). On the other hand, Pf*uis4*, Pf*plp1* and Pf*slarp* were amplified differentially between the two time points (Figure 7). This result was confirmed in a second replicate, as well as using an independent RNA preparation from the same batch of mosquitoes. However, because Pf*uis4* does not contain introns it is less suitable for assays where the isolated RNA will not be DNase-treated. On the contrary, the Pf*slarp* and Pf*plp1* genes have introns and can therefore be used in diagnostic assays that omit or cannot integrate this step.

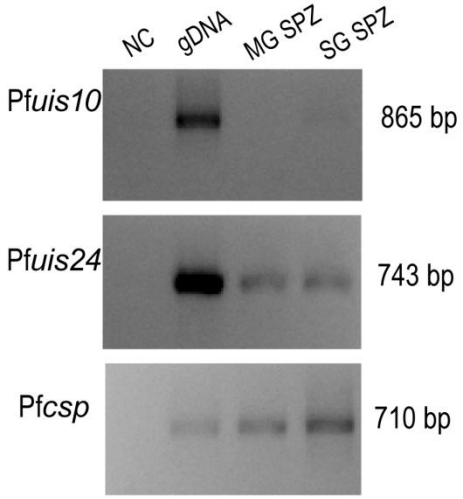

Figure 6. RT-PCR analysis of *P. falciparum* Pf*uis10* and Pf*uis24* transcripts. Templates were derived from gDNA (lane 2), dissected midguts 12 d sporozoites (lane 3, MG SPZ) and dissected salivary glands at 21 d pbm (lane 4 SG SPZ). Lane 1 is the negative control without added template (NC). The samples were amplified for 35 cycles. Molecular weight of the amplified fragments is indicated to the right

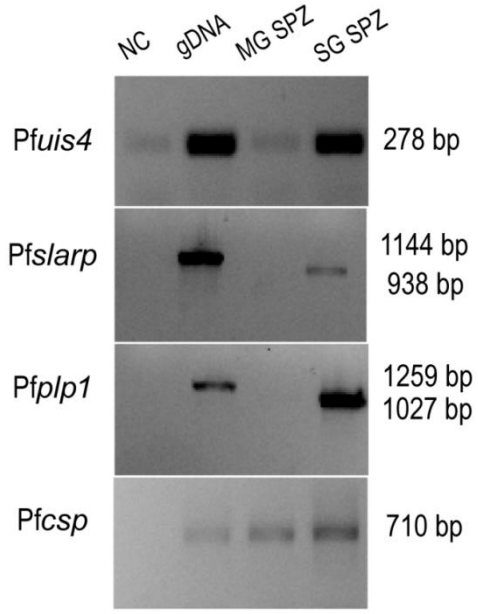

Figure 7. RT-PCR analysis of *P. falciparum* Pf*uis4*, Pf*slarp*, Pf*plp1* and Pf*csp* transcripts. Templates were derived from gDNA (lane 2), dissected midguts 12 d pbm (post blood meal), sporozoites (lane 3, MG SPZ) and dissected salivary glands 21 d pbm (lane 4 SG SPZ). Lane 1 is the negative control without template (NC). The samples were amplified for 35 cycles. Molecular weight of the amplified fragments is indicated to the right.

Development of a qRT-PCR TaqMan assay for infective mosquitoes

We next designed primers, end up with the optimal primers' and probes' concentrations (Table 3) after testing different combinations for each gene and used our qRT-PCR method for the measurement of normalized Pf*slarp* and Pf*plp1*transcript abundance in pools of whole mosquitoes. Both genes were found substantially up-regulated in the infective stage (Figure 8b,d). We next calculated the Relative Quantification (RQ) units for each sample and target gene ($RQ = 2^{-dCt}$, where dCt = (Ct target - Ct reference) and Ct is the threshold value). The data showed that the expression levels of Pf*slarp* were was 33.5 ±4.4 RQ units for the infective stage compared to 4.29 ± 1.7 RQ units for the non-infective pool (Table 4). Pf*plp1* expression levels were measured as 10.8.± 2.2 at the infective stage, and 0.43 ±0.21 at the non-infective stage. By using 1.23 or 10.34 RQ units as cut-off value for Pf*plp1* and Pf*slarp*, respectively, a sensitivity and specificity of 100% can be achieved in discriminating infective from non-infective mosquitoes.

Discrimination of samples can also be more easily achieved using only Pf*slarp* or Pf*plp1* Ct values (cut-off Ct = 33.0 for Pf*slarp* and Ct = 37.0 for Pf*plp1*) without the need of calculating normalized RQ units. This is supported by the fact that Pf*slarp* Ct values ranged from 27.1-29.6 in the infective samples, compared to values greater than 33.0 in the non-infective samples. Similarly, Pf*plp1* Ct values were at the range of 29.3-30.5 in the infective samples and practically non-detectable (Ct >37.0) in the non-infective pools (Figure 8a,c). Pf*csp* was used as a qualitative positive control rather than a quantitative normalizer in this case. Non-infected samples did not produce any Ct value.

We also investigated the sensitivity of the assay for the detection of *P. falciparum* sporozoites in the salivary gland. Infected mosquitoes were mixed with non-infected *A. gambiae* mosquitoes and then subjected to the TaqMan assay. Both Pf*plp1* and Pf*slarp* expression was unambiguously detected when the infective sample was mixed in a 1:20 or even 1:50 ratio with non-infected mosquitoes. Pb*plp1* was also detected in 1:100 ratio(Table 5).

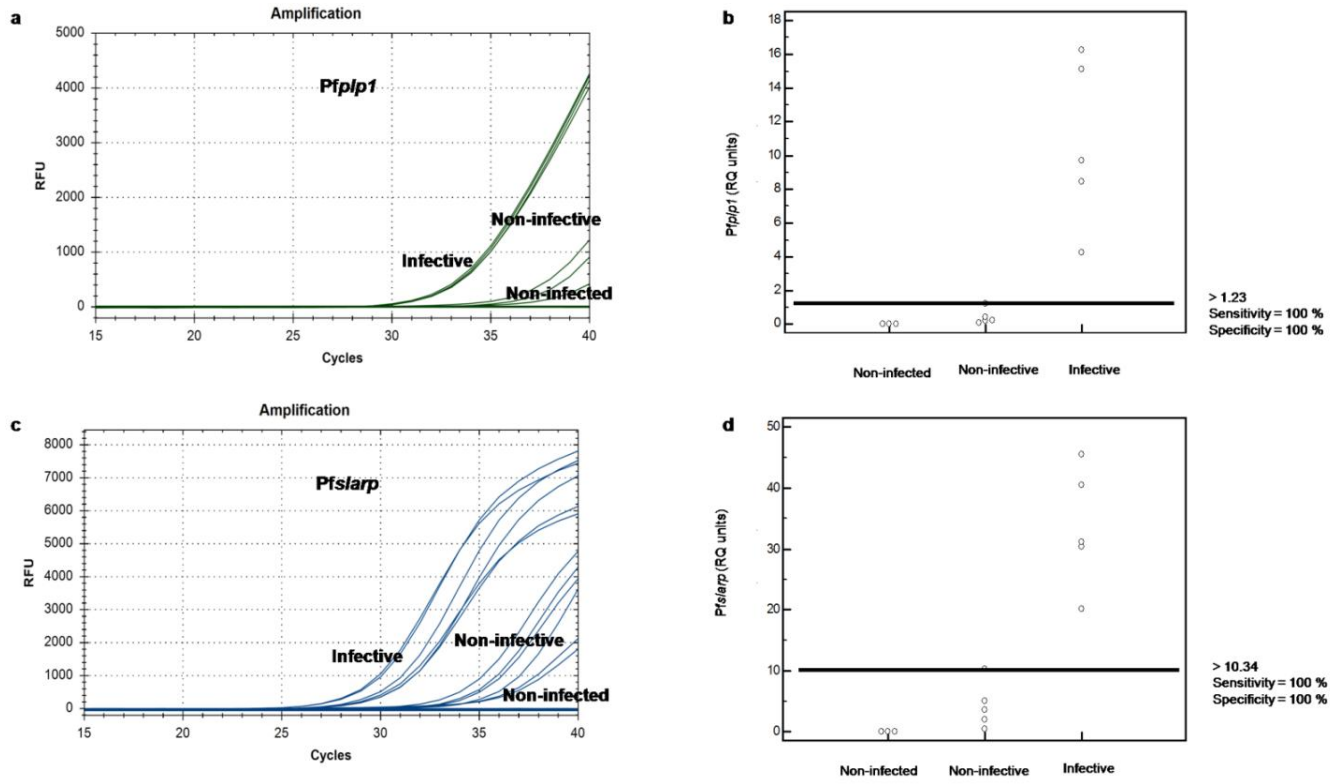

Figure 8. Differential diagnostic value of Pf*plp1* and Pf*slarp* expression. a, c: TaqMan amplification curves for Pf*plp1* and Pf*slarp* genes in representative infective, non-infective and non-infected samples. b, d: Expression levels of Pf*slarp* and Pf*plp1* in infective, non-infective and non-infected pools.

Gene	Infection status of samples	RQ (Mean ± SE)	95% CI	Ct
Pf*plp1*	Infective	10.8.± 2.2	4.6 - 16.9	29.3-30.5
	Non infective	0.43 ±0.21	0 - 1.01	>37.0
	Non-infected	Undetected	Undetected	Undetected
Pf*slarp*	Infective	33.5 ±4.4	21.3 - 45.8	27.1-29.6
	Non infective	4.29 ± 1.7	0 - 9.01	>33.0
	Non-infected	Undetected	Undetected	Undetected

Table 4. Descriptive statistics of Pf*plp1* and Pf*slarp* gene expression in infective samples compared to non-infective and non-infected samples.

Setup	SLARP-Singleplex	PLP1-Singleplex	SLARP-2plex	PLP1-2plex
Dilution				
1 infective in 10	**detected**	**detected**	**detected**	**detected**
1 infective in 20	**detected**	**detected**	**detected**	**detected**
1 infective in 30	**detected**	**detected**	**detected**	**detected**
1 infective in 50	**detected**	**detected**	**detected**	**detected**
1 infective in 100	**detected**	**detected**	**not detected**	**detected**

Table 5 Dilutions of infective with non-infected mosquitoes and testing of detection in SLARP-Singleplex, PLP1-Singleplex, SLARP-CSP-doubleplex and PLP1-CSP-doubleplex.

Discussion

The development of affordable and rapid diagnostic tests for malaria parasite infected mosquitoes, and more specifically for infective mosquitoes harbouring sporozoites in their salivary glands is of high priority for supporting evidence based vector control interventions(Vontas, Mitsakakis et al. 2016). Several assays have been devised for this purpose, including ELISA and PCR -based detection in dissected mosquito heads/salivary gland preparations(Fontenille, Meunier et al. 2001; Snounou and Singh 2002; Bass, Nikou et al. 2010; Marie, Boissiere et al. 2013). Here, we report the development of a dissection-free, one-step RT-qPCR method for detecting the infective stage of *Plasmodium falciparum* in its mosquito vector.

We initially took advantage of available proteomic and RNAseq data (PlasmoDB; www.plasmodb.org). Five genes were selected that were reported to be more highly expressed in sporozoites from the salivary glands compared to those of the midgut(Chattopadhyay, Rathore et al. 2003; Ishino, Chinzei et al. 2005; Silvie, Goetz et al. 2008; Talman, Lacroix et al. 2011). We also investigated expression of the *uis* genes which were originally identified based on the up-regulation of their transcripts in salivary gland sporozoites. Dissected midguts and salivary glands of mosquitoes infected with the rodent model parasite *P. berghei* were analysed using semi-quantitative RT-PCR. The two genes Pb*slarp* and Pb*plp1* were expressed at considerably higher levels in sporozoites from the salivary gland compared to those of the midgut (Figure 3) and three of the *uis* genes fulfilled our selection criteria (Figure 4). Next, we tested these genes using *P. falciparum* infected samples. The results of this analysis suggested that transcripts of the genes Pf*slarp* and Pf*plp1* as the most suitable candidates for a RT-PCR-based assay (Figure 7), and a qRT-PCR TaqMan assay was developed and optimized (Figure 8).

Our results show that both selected genes present substantially higher expression levels in infective compared to non-infective pools, although each gene showed specific advantages. Pf*plp1* could be assessed in a 2-plex format together with the normalizer Pf*csp,* thus minimizing time and amount of sample needed for reaction preparation. Additionally, Pf*plp1* showed less variability between replicates compared to Pf*slarp* (Figure 8d). Pf*slarp* presented generally higher expression levels but could be reliably assessed only in single-plex format without compromising sensitivity. The assay needs to be further validated with naturally infected field caught samples since our assays employed laboratory infected mosquitoes. However, the median infection intensity of assayed mosquitoes was 2, which is similar to levels found in field infected mosquitoes. Furthermore, our assay can only be applied with *P. falciparum* infected samples, at present, but can conceptually be extended to other important *Plasmodium* parasites such as *P. vivax*. Future work will focus on the development of similar assays for additional epidemiologically relevant *Plasmodium* species, should the access to appropriate samples and resources becomes possible.

In conclusion, our novel assay represents significant improvements in detecting infective *P. falciparum* mosquitoes. The assays do not require dissection of mosquitoes nor is DNase-treatment necessary. The sensitivity was high as we could detect parasite transcripts in samples containing a mix of infected mosquitoes with non-infected at a 1:100 ration, allowing for pools of individual mosquitoes to be tested. We also found that *P. falciparum* infected mosquitoes kept in RNAlater, at ambient temperature, for several days produced good quality RNA suitable for this assay. This is very important for the future development of diagnostic kits, given the possibility of using lyophilised RT-PCR pellets (transcriptase and probes), which can be manufactured and shipped without a cold chain. Furthermore, the assay is

flexible and provides the option of multiplexing either with other parasite markers or even mosquito transcript markers such as insecticide detoxification genes (expressed primarily in abdomens) that are associated with insecticide resistance. Thus, this novel assay represents an ideal candidate for incorporation in automated diagnostics platforms for mosquito vector surveillance (Bhatt, Weiss et al. 2015).

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