Factors Triggering *Plasmodium* Development Following *Anopheles* Salivary Gland Invasion

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ABSTRACT

Malaria parasites undergo a sequential series of developmental changes as they traverse and invade various tissues of both vertebrate and invertebrate hosts. These developmental changes are reflected in the parasites' morphology, infectivity, gene expression patterns, as well as levels. Midgut and hemolymph sporozoites are completely different from those of salivary gland ones in that they are less capable for infecting their hosts and are less motile. The mosquito factors triggering those developmental changes during, and post salivary gland invasion are largely unknown. To identify and characterise mosquito factors that trigger those essential developmental changes in *Plasmodium berghei* and strengthen sporozoite development to infect their mammalian host, a UIS4:*mCherry*-containing line of *P. berghei* (*Pb*ANKA-*Cherry*-2204c1) that only has visible *mCherry* expression following salivary gland invasion, was treated *in vitro* with adult female mosquito tissue homogenates. The data obtained from using female *Anopheles stephensi* salivary gland homogenate shows the expression of *mCherry* protein as red fluorescence in about 70% of treated sporozoites *in vitro*. *Anopheles stephensi* midgut homogenate (non-specific tissue), and *Aedes aegyptie* salivary gland homogenate (non-vector salivary gland) were used as well as UIS4, UIS7, and *mCherry* genes expression were compared with midgut sporozoites. Treated sporozoites have a transcription profile of the tested genes like that of salivary gland sporozoites. Although the variables causing *Plasmodium* development following mosquito salivary gland invasion may not be influenced by mosquito species, it does show tissue specificity. As a result, the salivary gland's function as a gateway and invasion is the most crucial and specific phase in disease transmission.

Keywords: *Anopheles; Plasmodium*; Salivary gland.

INTRODUCTION

Globally, according to the latest report on Malaria revealed from WHO in 2020, there were an expected 241 million cases of malaria in addition to 627000 deaths worldwide. This represents around 14 million extra infected cases and 69000 extra deaths in 2020 if compared with 2019. About two-thirds of these additional deaths (47000) were due to shortage of malaria prohibition, diagnosis and reme-diation during the pandemic, (World Health Organization, 2020).

Integrated strategies for any malaria control program have sparked interest in several new approaches including antimalarial drugs (Insight Review Articles 686, 2002), renewed efforts to find transmission vaccines (Todryk and Hill, 2007), and the development of genetically modified mosquitoes (GMMs) to reduce the population size or to replace the current populations with vectors unable to transmit the disease (Marshall and Taylor, 2009). Transgenic mosquitoes should be viewed as a potential factor in the future goal of disease control. The discovery of refractory genes for rodent malaria and the gene drive system raises the possibility of similar achievements for human malaria in mosquito vector species (Marshall and Taylor, 2009; Isaacs *et al.*, 2012; Gantz *et al.*, 2015).

The understanding and targeted manipulation of mosquito-*Plasmodium* (*P*) interactions could be crucial for improving production and development of whole *P. falciparum* sporozoite vaccines and infectious sporozoites. Anti salivary gland antibodies and certain lectins are capable of interfering with sporozoite invasion of salivary gland. The salivary gland cells are the second insect epithelial barrier facing the parasite (Smith and Jacobs-Lorena, 2010).

The *Plasmodium* sporozoites should migrate after being released from oocysts into the hemolymph to the basal lamina of the salivary glands where they recognize, attach, invade, develop, and finally emerge from the salivary gland cells to the salivary duct. The majority of sporozoites remain in these cavities and only a few enter the salivary gland duct. Mosquito's salivary gland invasion by sporozoites is one of the most important events of the malaria cycle (Pimenta, *et al.*, 1994; Frischknecht *et al.*, 2004).

Recent technological advances have improved the understandings of the *P.* parasite including the less known sporozoites stage at the molecular level. The accomplishment of the *P. falciparum* genome sequence allowed the analysis of gene expression at different stages of the parasite life cycle (Le Roch *et al.*, 2003). Approximately 2000 genes are expressed in salivary gland sporozoites, 500 of which are expressed at high levels and around 100 of these are not expressed at significant levels in blood stages. About 123 genes were identified to be expressed in *P. berghei* sporozoites (Rosinski-Chupin *et al.*, 2007).

Plasmodium sporozoites are formed in the oocysts in the mosquito midgut, but full infectivity coincides with colonization in the mosquito salivary gland. This maturation process is associated with the upregulation of specific upregulated infective sporozoites (UIS) genes (Matuschewski *et al.*, 2002). The analysis of gene structure and/or gene expression data confirmed that the mRNA levels may vary not only between midgut and salivary gland sporozoites but also during the storage of the sporozoites in the salivary gland (Rosinski-Chupin *et al.*, 2007).

The circumsporozoite protein (CSP) is one of the most important micronemal surface proteins and play an important role in sporozoite development within oocysts and essential during the mosquito salivary gland invasion (Combe *et al.*, 2009; Steinbuechel and Matuschewski, 2009; Ménard *et al.*, 1997). Although UIS4 is highly expressed by sporozoites in the mosquito salivary glands (Matuschewski *et al.*, 2002), the genetic studies in the rodent malaria parasites have shown that it is required only after transmission to the vertebrate host during liver stage development (Mueller *et al.*, 2005; Tarun *et al.*, 2007). UIS4 of rodent malaria parasites and UIS4 of the human malaria parasite *P. falciparum* show 34% amino acid sequence identity. Rodent malaria parasites such as *P. berghei* are excellent models to study *P.* liver-stage and pre-erythrocytic immunity (Mueller, *et al.*, 2005).

In the current study, to investigate which factor(s) may affect the midgut treated sporozoites *in vitro* and enhanced their development to mimic and act as post invasion salivary gland sporozoites, large numbers of sporozoites were used for addressing the nature of the factors associated with salivary glands responsible for triggering these major developmental changes in the tested mosquitoes.

MATERIALS AND METHOD

Mosquito rearing and infection

Anopheles stephensi SDA 500 were used throughout this study, and it was chosen because it is particularly vulnerable to *Plasmodium falciparum* (Feldmann and Ponnudurai, 1989). All life stages were reared as adult at the Institute for Bioscience and Biotechnology researches (IBBR) under standard conditions of 28 ±2 °C, 80% relative humidity (RH) and a 12-hour light/dark cycle. Larvae were fed pulverized fish food (TetraMin® Tropical Flakes) ad libitum while adults had continuous access to a 10% sucrose solution. Mated adult females were blood-fed on 4-6 week old BALB/c female Mus musculus. Three to four days old female *Anopheles stephensi* SDA 500 were allowed to feed on anaesthetized BALB/c mice infected with *Pb*ANKA-*Cherry*-2204cl with a parasitemia of 25-30%. Fed mosquitoes were maintained on a solution of water containing 8% w/v dextrose solution and 0.05% w/v para-amino benzoic acid at 24 °C± 2 and 70% RH in 12: 12 light / dark cycles.

Plasmodium berghei:

The transgenic *Plasmodium berghei* line *Pb*ANKA-*Cherry*-2204cl was obtained from the Leiden Malaria Research Group (Leiden University Medical Center, Leiden, Netherlands). *Pb*ANKA-Cherry 2204cl contains a *mCherry* expression cassette flanked by the 5' and 3' UTRs of *P. berghei* UIS4 integrated into the 230p locus of *P. berghei* GIMO *Pb*ANKA. *mCherry* expression is only detectable in sporozoites after they have entered the salivary glands of the mosquito host. A description of *Pb*ANKA-*Cherry*-2204c1 can be found in the Rodent Malaria genetically modified Parasites Data Base (RMgm-1339) (Khan *et al.*, 2013; Janse *et al.*, 2011).

Mosquito dissection:

The infected Mosquitoes (10-14 days post infection and 15-22 days post infection) were anesthetized by chilling at 4 °C±2, until immobilization. quickly surface sterilized with 70% ethanol and washed in 1 X Phosphate Buffered Saline and kept cold on ice prior to dissection. mosquitoes were dissected in RPMI 1640 (1640 medium, Corning, Life Sciences, Tewksbury, Massachusetts). Midguts and salivary glands were dissected as described (Kennedy *et al.*, 2012) (Coleman *et al.*, 2007) and placed in 50µl of cold RPMI 1640.

Sporozoites isolation

The pre-invasion and the post-invasion sporozoites were isolated from the infected mosquitoes after 10-14- and 15-22-days post infection respectively by dissecting midguts and salivary glands of infected mosquitoes (30-50) and placed in 50 µl of cold RPMI. Free sporozoites were obtained by gentle grinding the pooled midguts and salivary glands separately in each batch in 100 µl RPMI 1640 with a plastic pestle. Released sporozoites were counted and kept on ice for immediate use or cryopreservation (Singh *et al.*, 2016).

Cryopreservation of *P. berghei* sporozoites

A 10,000 sporozoites/µl suspension was prepared (in RPMI 1640:cryogenic solutions (CryoStor Cell CS2, Sigma Lot # MKBZ6988V) (1:3) and cryopre-served in pre-chilled cryogenic vial (Thermo Scientific # 3744-BR Matrix) 250,000 sporozoites per vial (Singh *et al.*, 2016).

In vitro treatment:

The fresh pre-invasion midgut *Pb*ANKA-*Cherry*-2204c1 sporozoites were kept on ice for 5 min for thawing then divided according to the number of control and treated samples, for negative control; add 50 µl RPMI 1640 media to pre-invasion midgut sporozoites. For the treatment groups: group (1) 20 female *Anopheles stephensi*-salivary glands crude extract were added in 50 µl RPMI media, group (2) 10 female *Anopheles stephensi*-midguts crude extract was added in 50 µl RPMI media, group (3) 20 female *Ae. Aegypti*-salivary glands crude extract was added in 50 µl RPMI media. All sample groups were incubated at 25 °C ±2 for 4 hrs. for further investigations. To investigate which factor(s) may be affect the treated sporozoites *in vitro* and enhanced their development, large numbers of sporozoites were used for further molecular investigations. The previous experiment was repeated 3 times using a larger number of cryopreserved *Pb-mCherry*-@UIS4 transgenic pre-invasion midgut sporozoites ($\approx$200-250 x 10^3) were incubated under the same conditions for both treated and untreated samples.

Sporozoites visualization and counting

Negative control and group (1) treated samples' sporozoites were mounted on a glass slide under coverslips in VECTASHIELD® Mounting Medium (Vector Laboratories, Burlingame, CA) and observed with a Zeiss Axio Imager.A1 under phase contrast and red fluorescence illumination to visualize *mCherry*. The total sporozoites number was counted for all samples of the examined groups, as well as *mCherry*-

expressing sporozoites (at least 5-fields for each slide, 3-slides from each tube and the whole experiment was repeated at least 3 times for each case). To confirm the data obtained from preliminary microscopic observations, further molecular investigations using the quantitative real time PCR were undergone.

Quantitative real time PCR

To investigate which mosquito components are responsible for sporozoite maturation *in vitro*, cryopreserved midgut sporozoites were treated with crude extracts of *An. stephensi* salivary gland, *An. stephensi* midgut (different mosquito tissue), and *Aedes aegyptie* salivary gland (non-vector) and incubated at 25°C ±2 for 4 hrs compared to the midgut control group. Whole sporozoite-RNA were isolated from each group separately. Quantification Real Time Polymerase Chain Reaction qRT-PCR was performed for *mCherry*, UIS4 and UIS7. ΔCt values were norm-alized to CSP and the fold change were calculated against control groups.

Total RNA was extracted from the experimental and control groups' samples using Direct-ZolTM RNA MicroPrep suggested protocol. The first cDNA strand was synthesized using SuperScriptIII (Invitrogen, cat#1821203) suggested protocol using random Hexamers. The qPCR technique was done for all samples using Cyber Green GoTaq Master Mix, several primer sets, and Applied Biosystems (7300 Real Time PCR System) machine was used for the run. The results obtained from real time PCR data were analyzed using the comparative CT method and comparing the change in fold change (Schmittgen and Livak, 2008).

Western blotting

Total protein was extracted from the experimental and control groups' samples by homogenising the sporozoites with 0.1 l of 1X protease inhibitor cocktail (SC248229A; Thermo Fisher Scientific, Waltham, Massachusetts). The extracted protein samples were processed for Western Blotting to identify the *mCherry* protein using the Invitrogen iBlot 2 PVDF MiniStacks sandwich (Thermo Fisher Scientific, Waltham, Massachusetts) protocol to transfer protein from gel to pre-activated PVDF membrane at 20V, 0.9A for 6 minutes. A rabbit polyclonal *mCherry* antibody (ab167453; abcam, Cambridge, UK) was the primary antibody (1:1000 in 3% milk-TBS) and an anti-rabbit IgG antibody-alkaline phosphatase conjugate (S373B; Promega, Madison, Wisconsin) was the secondary antibody (1:10^4 in 3% milk-TBS T). Western blot membranes were stabilized with alkaline phosphatase substrate (S3841; Promega, Madison, Wisconsin) for ~10-20 minutes before being viewed with the Java image processing tool ImageJ 1.48. (Schneider *et al.*, 2012).

Statistical analysis

The data were statistically evaluated for normality using the Shapiro-Wilk normality test at a significance level of $p \leq 0.05$. According to this test, which indicates that the data are parametric, ANOVA was carried out. The data described in terms of Mean ± standard error (Mean ± SEM). Differences among treatments were evaluated using according to Duncan's multiple range tests (DMRTs).

RESULTS

The factors that influence *Plasmodium* development and maturation, following *Anopheles* salivary gland invasion, revealed a diversity of responses that may not be affected by mosquito type.

Effect of *Anopheles stephensi* salivary gland homogenates on treated sporozite

On freshly isolated PbANKA-Cherry-2204c1

Interestingly, the most striking observations appeared after the examination of the treated sporozoites under both the white and red fluorescence illumination showed several sporozoites turned to red fluorescence which indicates the expression of the *mCherry*@UIS4 in the treated sporozoites samples (Figure 1A-B). The previous results were compared to freshly isolated pre-invasion midgut sporozoites (negative control, Figure 1C-D), which were isolated and cultured under the same conditions but without any mosquito salivary gland tissues. Following incubation, over 70% of the treated sporozoites, group1 samples, were red fluorescent, compared to almost 9% of the untreated sporozoites samples (Table 1).

On cryopreserved PbANKA-Cherry-2204c1

Salivary gland homogenates, from *Anopheles stephensi*, displayed an effect on cryopreserved *Pb*ANKA-*Cherry*-2204c1 (Table 1). The data obtained revealed that 66.81 % of cryopreserved pre-invasion midgut sporozoites of the treated group1 samples became red fluorescent post-incubation compared to only 9.20 % of the untreated sporozoites post incubation. However, comparison between freshly isolated *Pb*ANK*A-Cherry*-2204c1 versus cryopreserved sporozoites, expressed as total and red fluorescent sporozoites counts were reported in Table (1).

The total and red fluorescent sporozoite counts for all defined sub-groups exhib-ited a significant difference ($p \leq 0.001$) regardless of whether the sporozoites were fresh or previously cryopreserved under particular protocols (Table 1). Meanwhile, a significant varia-tion in total sporozoite count ($p \leq 0.004$) was observed between fresh and cryopreserved sporozoites. However, no significant variation in its count percentage was observed before and after treatment, indicating that the *Anopheles steph-ensi* salivary gland tissue extracts had no effect.

Effect of different mosquito tissue or species homogenates on target gene expression

Anopheles stephensi salivary gland homogenates
The expression of tested mRNA *mCherry*, UIS4 and UIS7 genes from the *Anopheles stephensi* salivary gland homogenates treated *Pb*ANKA-*Cherry*-2204c1 transgenic sporozoites, reveals surprising high significant ($p \leq 0.0001$) increase post incubation. ΔCt values were normalized to CSP and the fold change were calculated against control groups (Figure 3A).
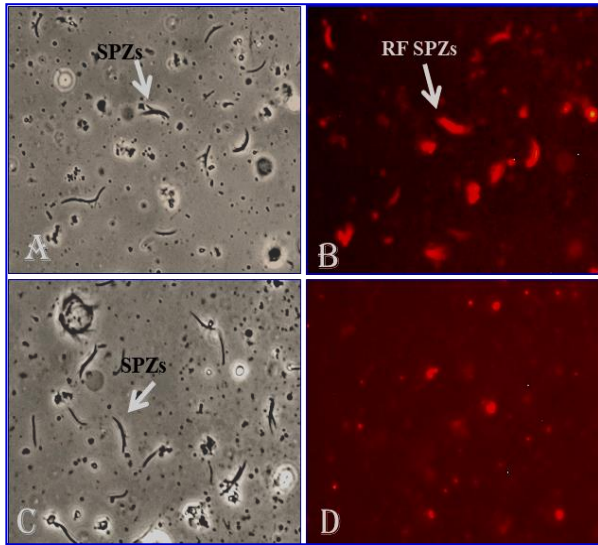
Figure (1): The effect of *Anopheles stephensi* salivary gland (SG) homogenates on the maturation of freshly isolated *Pb-mCherry*@UIS4 transgenic sporozoites (SPZs) 4 hours after incubation *in vitro*. A-B, treated group, under phase and fluorescent microscope in which several sporozoites turned to red fluorescence (RF SPZs, arrow); C-D, control group observed under the same conditions.

Effect of Anopheles stephensi Midgut homogenates

The expression of tested mRNA genes from *Pb*ANKA-*Cherry*-2204c1 transgenic sporozoites, were did not reveal any significant (*p* values >0.06) increase post *Anopheles Stephensi* midgut homogenates treatment compared with untreated control group (Figure 3B).

Effect of Ae. aegyptie salivary gland homogenates

The effect of non-vector mosquito salivary gland on *mCherry*, UIS4 and UIS7 mRNA gene expression is displayed in Figure (3C). Sporozoites treated with a crude extract of *Aedes aegypti* salivary gland (group 3) revealed a significant (*p* ≤0.0001) increase in the fold change in the expression of the same targeted genes compared to the negative control samples (Figure 3C).

However, all treatments together on *mCherry,* UIS4 and UIS7 mRNA gene expression in the transgenic sporozoites *in vitro* showed an increase in the fold change of the tested genes expression in samples treated with *Anopheles stephensi* and *Ae. aegypti* salivary gland homogenates compared to the control.
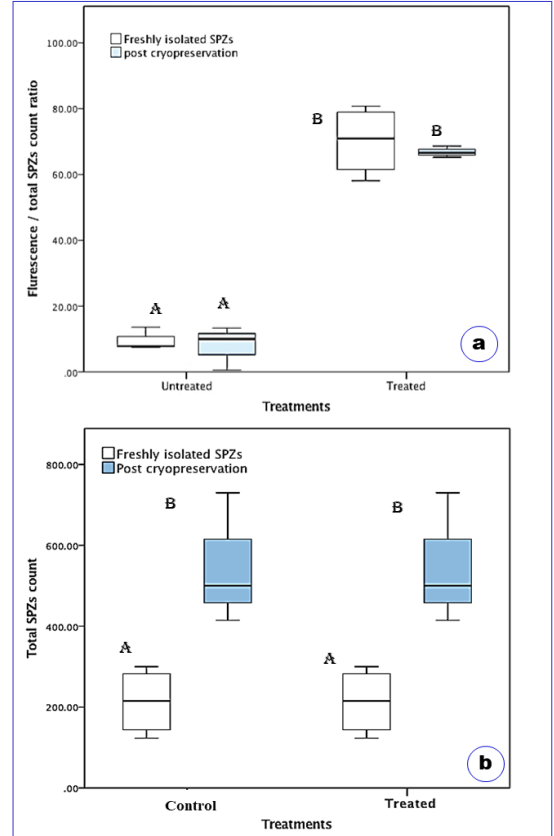

Figure (2): Effect of *Anopheles stephensi* salivary gland homogenates on cryopreserved *Pb*ANKA-*Cherry*-2204c1 transgenic sporozoites maturation 4hrs post-incubation *in vitro*. a; the ratio between total and fluorescence counts; b, only the total Boxes with different letters are significantly different at *p* ≤0.05 according to Duncan's multiple range tests (DMRTs).

Table (1): Table (1) Total sporozoites count, percentage, fluorescence, fluorescence/count ratio, and mortality rate recorded 4 hrs. post incubation for the treated samples (Midgut *Pb*ANKA-*Cherry*-2204c1 transgenic sporozoites treated with the crude extract of *An. stephensi*-salivary glands), and the untreated control.

Measured parameters	Treatments[†]	Sporozite used		F-ratio	*p*-value[†]
		Freshly isolated	Post Cryopreservation		
Total sporozoites count	1	190.00 ± 59.66 [a]	535.00 ± 68.89 [b]	8.36	0.004[**]
	2	198.25 ± 59.66 [a]	496.67 ± 68.89 [b]		
Sporozoites count before incubation	1	213.25 ± 60.74 [a]	548.33 ± 70.14 [b]	8.69	0.004[**]
	2	213.25 ± 60.74 [a]	548.33 ± 70.14 [b]		
Fluorescence sporozite count after incubation	1	16.75 ± 28.32 [a]	48.67 ± 32.70 [ab]	19.87	≤0.001[***]
	2	135.75 ± 28.32 [b]	330.33 ± 32.70 [c]		
Fluorescence sporozite count after incubation (%)	1	9.20 ± 3.36 [a]	7.94 ± 3.88 [a]	93.15	≤0.001[***]
	2	70.18 ± 3.36 b	66.81 ± 3.88 [b]		
Ratio of Sporozite count before and after incubation (%)	1	89.54 ± 2.96 [a]	97.41 ± 3.42 [a]	1.176	0.367 [NS]
	2	93.44 ± 2.96 [a]	90.41 ± 3.42 [a]		
Mortality rate (%)	1	23.25 ± 12.81 [a]	13.33 ± 14.80 [a]	1.499	0.274 [NS]
	2	15.00 ± 12.81 [a]	51.67 ± 14.80 [a]		

[†]1, control group, no treatment applied; 2, treated group. Data are in mean±SE; [†]*p*-values with [**] are significant difference; values with [***] are highly significant; values with NS are not significantly different; Means followed by different letters are significantly different at *p*≤0.05 according to Duncan's multiple range tests (DMRTs).

On the other hand, there is no significant difference in the fold change was noticed according to the mosquito species (Figure. 3D). The data revealed that the samples treated with *Anopheles stephensi* salivary gland shows the highest increasing in the fold change. The samples treated with the non-vector *Ae. aegypti* salivary gland tissue were ≈ 1.5 times lower than the sporozoites treated with vector salivary gland tissue. Meanwhile, the samples that were treated with different tissues from the vector tissue didn't show a significant increase in the fold change and have no significant effect on the treated sporozoites (Figure. 3D).

These data indicate that pre-invasion sporozoites, following exposure to mosquito salivary gland tissue, shows evidence of maturation to post-invasion sporozoites as reflected in the expression of the post-invasion salivary gland *mCherry@UIS4* gene regardless the vector competency. Meanwhile, the data obtained from western blot to detect *mCherry* protein expression (Figure 3E), shows a significant difference ($p \leq 0.05$) between the pre-invasion and the post-invasion samples within *mCherry*.

DISCUSSION

Reducing the rate of re-infection in humans will be critical to eradicate malaria, and that the natural population bottlenecks in mosquito stages of *Plasmodium* are vulnerable points to disrupt the parasite's life cycle and prevent disease transmission (Yenkoidiok-Douti *et al*., 2020). In this study, two mosquito factors that may play an important role in sporozoites maturation were evaluated *in vivo* using the *P. berghei* model. The obtained results revealed that although the factors triggering *P.* development following mosquito salivary gland invasion might not be affected by mosquito species, it has tissue specificity.

Plasmodium sporozoite invasion of mosquito salivary glands is a vital step in the success of malaria transmission, as sporozoites boost infectivity and enable transmission to vertebrate hosts during blood feeding. A complete understanding of the factors that affect sporozoites development and expression of different genes in salivary gland invasion, the main target of our study, could be used to reduce vector-borne infections (Mueller *et al*., 2005).

The *mCherry@Pb*-UIS4 transgenic sporozoites were genetically modified which expresses RFP only inside the salivary gland of the infected mosquitoes that refers to the sporozoites development and provides strong positive control for the difference between midgut and salivary gland sporozoites *in vivo*, similar results were reported by Lin *et al.,* (2011) and Hopp *et al*., (2015). The *Pb*ANKA-*Cherry*-2204c1 transgenic sporozoites were genetically modified which express RFP only inside the salivary gland of the infected mosquitoes which refers to the sporozoites development (Hopp *et al*., 2015).

The preliminary notice from the microscopic examin-

ation of the treated *mCherry@Pbuis4*-2204 (RMgm-1339) *P. berghei* pre-invasion sporozoites with *Anopheles stephensi* salivary gland extract after 4 hrs. incubation revealed that the treated sporozoites are genetically developed to be similar to the post-invasion sporozoites. The detected expression of *mCherry* protein as a red fluorescence only in the treated sporozoites may be referred to a specific signal in the salivary gland extract triggered the sporozoites maturation in a similar manner like the post invasion salivary gland sporozoites.

Comparing the data obtained from the microscopic examination of the cryopreserved sporozoites with the freshly isolated ones under the same treatment conditions clearly implies the advantages of the cryopreservation in using a huge sporozoites number with no significant effect on sporozoites recovery or viability. This finding confirms the data which were reported by (Singh *et al*., 2017). The results show a critical increase in the RNA expression in the treated samples compared to the negative control sporozoites for UIS7, UIS4 & *mCherry*. These results confirm those of Matuschewski *et. al*. (2002) and Rosinski-Chupin *et al*., (2007). The critical increase in the RNA expression of the treated samples support the present data obtained from the microscopic examination which might be resulted from the genetically configurations for tested genes obtained from the QPCR analysis of the treated sporozoites.

The current observed data obtained from QPCR analysis showed an important increase in the UIS4, *mCherry* and UIS7 genes in the sporozoites treated with *Anopheles stephensi* salivary gland extract as salivary gland sporozoites. The demonstrated critical upregulation of tested genes post incubation reflected the maturity and capability of the treated sporozoites to be infective. These findings confirm the results obtained from the microscopic examination which gave an indication to a specific signal in the salivary gland extract enhanced the sporozoites maturation.

The results revealed that there is a significant increase in the fold change in tested genes' expression among the *Anopheles stephensi*-salivary glands treated samples comparing to the negative control group, the sporozoites could not reach the same level of the gene expression as the salivary gland sporozoites positive control. This non-significant decrease in the fold change in gene expression of the treated sporozoites comparing to the positive groups may be attributed to the differences between the *in vitro* conditions and the in vivo ones as temperature, incubation period, or mosquito physiological state, the present finding confirm that of Touray *et al*. (1992) who stated that the salivary gland sporozoites vertebrate infectivity is associated with the sporozoites residence in the mosquito salivary glands. The changes in genes expression essential for the sporozoites development in vertebrate cells related to the salivary gland sporozoites infectivity increases from day 14 to day 18 post blood meal (Rosinski-Chupin *et al*., 2007). The previous conclusion might be explain the results from QPCR in
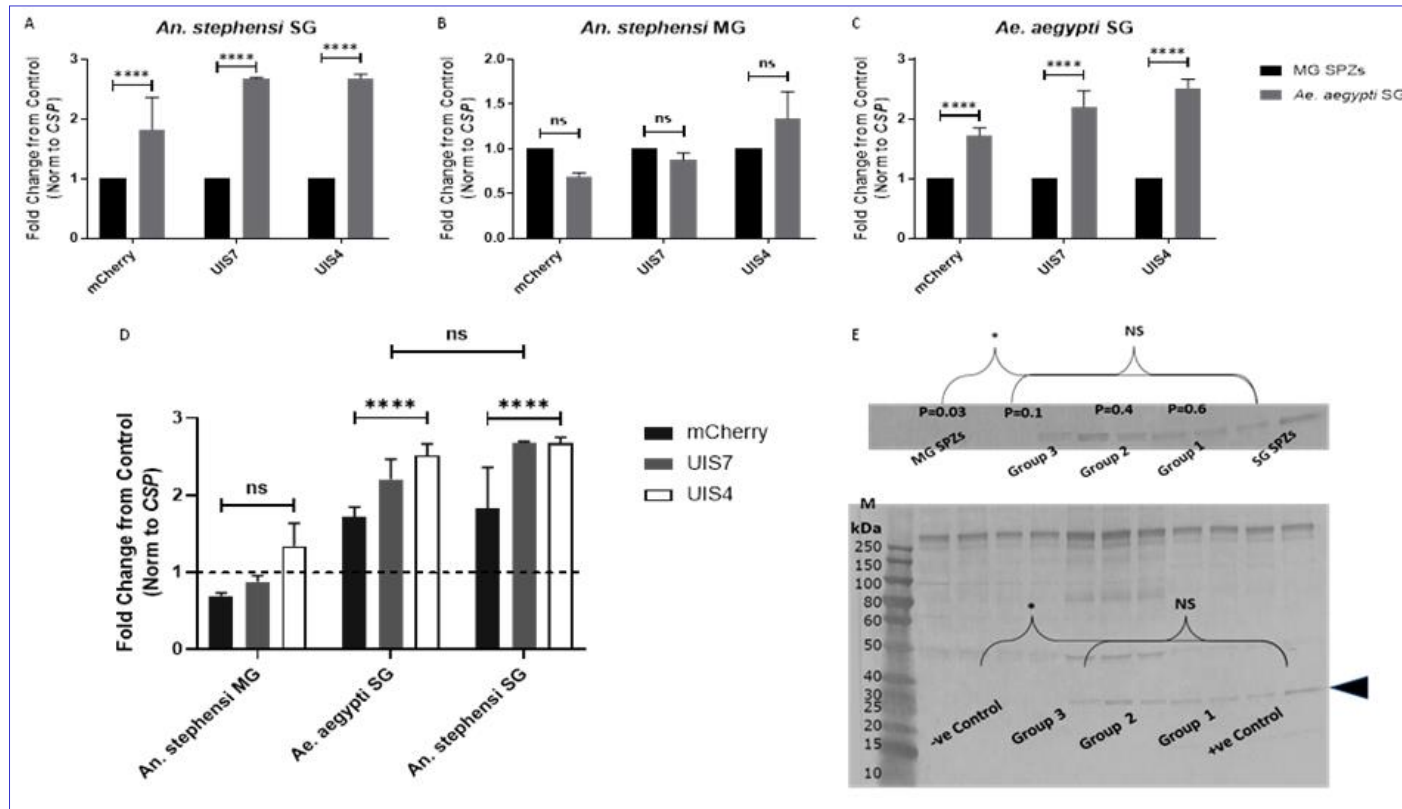
Figure (3): *In Vitro* effect of different mosquito tissue or species homogenates on mCherry, UIS4 and UIS7 mRNA gene expression in *Pb*ANKA-*Cherry*-2204c1 transgenic sporozoites. A, *Anopheles stephensi* salivary gland homogenates; B, *Anopheles stephensi* midgut homogenates; C, *Aedes aegypti* salivary gland homogenates; D, gene expressions for all treatments together; E, Western blot analysis of salivary gland and midgut of sporozoites *in vitro*. Midgut (MG), Salivary gland (SG), sporozoites (SPZs). ΔCt values were normalized to CSP and the fold change were calculated against control groups. **** , data are significant differences at *p*≤0.0001 using one way analysis of variance (ANOVA).

this work which showed a small difference between the treated samples and the salivary gland sporozoites as positive control. This non-significant decrease in the fold change of gene expression is likely to result from the condition, incubation period and the temperature difference between the *in vivo* and *in vitro*.

Several properties differ between midgut and salivary gland sporozoites, but it is not known whether the developmental changes are time and/or tissue dependent nor which signaling factors are involved. The salivary gland treated sporozoites, the negative control and positive control QPCR presented here indicates that at least not only the tissue but also the time spent in salivary gland significantly influences the level of expression of individual genes. The obtained result confirms the strong relation between the sporozoites motility, infectivity and other gene(s) differences and not only the mosquito tissue type but also the time spent in the mosquito salivary gland This conclusion affirms that of Vanderberg, 1975.

CONCLUSION

The current decrease in *Anopheles* population density in African metropolitan centres, along with an increase in culicine mosquitoes, may tilt the balance in favour of parasite adaptation to culicines. Such adaptation could have significant implications for public health and therefore more attention to avoid such possibility should be paid. The previous findings conclude that salivary gland homogenates stimulated sporozoites development from pre to post invasion. So, the factors triggering the *Plasmodium* development following *Anopheles* salivary gland invasion may be not affected by mosquito species. Based on conclusion, more investigations for other factors including biochemical and molecular levels are recommended.

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دراسة امكانية غز البلازموديوم و طفيلي الملاريا للغدد اللعابية المستأصلة من بعوض *Anopheles* و النامية على وسط غذائي مناسب

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الملخص العربي

تخضع طفيليات الملاريا لسلسلة متتابعة من التغيرات التنموية لأنها تغزو أنسجة مختلفة في كل من الفقاريات واللافقاريات. تظهر هذه التغييرات التنموية مورفولوجياً، والقدرة على العدوى، و مستويات التعبير الجيني. يختلف اسبوروزويتات المعى المتوسط و الهيموليمف تمامًا عن الغدد اللعابية من حيث أنها أقل حركة و غير قادرة على إصابة العائل الفقاري. لتحديد وتوصيف طبيعة عوامل البعوض التي تحفز تلك التغيرات التنموية الأساسية في دورة حياة البلازموديوم وتعزز تطور الاسبوروزويتات لإصابة مضيفها الفقاري، تم معالجة نوع من البلازموديوم المعدل وراثياً (*Pb*ANKA-*Cherry*-2204c1) و الذي لا يتحول لونه الى الاحمر الفلوري الا بعد غزوه الغدد اللعابية للبعوض) مع انسجة مختلفة من البعوض. تُظهر البيانات التي تم الحصول عليها من استخدام مستخلص الغدد اللعابية لبعوض الأنوفيليس الناقل لملاريا الفئران الى تحول 70 ٪ من الاسبوروزويتات المعالجة في المختبر الى اللون الاحمر الفلوري. ولمعرفة طبيعة عوامل البعوض المحفزة لنمو الاسبوروزويتات و تحوله الى الطور المعدي مباشرة بعد غزوه للغدد اللعابية. تم استخدام مستخلص المعي المتوسط لنفس البعوض الناقل كنسيج مختلف، و مستخلص الغدد اللعابية لبعوض الايديس كغير ناقل لملاريا الفئران. و بقياس تعبير بعض الجينات المستخرجة من المجموعات المعالجة بالانسجة المختلفة و مقارنة نسبتها مع مثيلتها المستخرجة من اسبوروزويتات غير معالجة معزولة من المعي المتوسط او من الغدد اللعابية من بعوض معدي معملياً. اشارت النتائج الى ان التعبير الجيني للاسبوروزويتات المعالجة بمستخلص الغدد اللعابية يشابه الى حد كبير تلك الخاصة بالاسبوروزويتات المعزولة من الغدة اللعابية. تشير النتائج إلى أنه على الرغم من أن العوامل المسببة لتطور البلازموديوم قد لا تتأثر بنوع البعوض ، إلا أن لها خصوصية في نوع النسيج. وبالتالي فإن الغدد اللعابية تلعب دوراً هاماً كبوابة وغزوها هو الخطوة الأكثر أهمية و تحدياً في انتقال المرض.