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Purification, expression and partial characterization of glutathione s-transferases (GSTs) from three different strains of *Aedes albopictus* (Diptera: Culicidae)

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Abstract. Glutathione S-transferases (GSTs) are multifunctional enzymes involved in detoxification of xenobiotic compounds in many insect groups. This work investigated the expression of GSTs in *Aedes albopictus* by using affinity chromatography and proteomic analysis. The study compared the expression of GSTS isoforms in larvae of three *Ae. albopictus* strains (susceptible (VCRU) strain, field Kuala Lumpur (KL) strain and laboratory permethrin-treated strain). The study demonstrated that the expression of sixteen isoforms (GSTS1-1, GSTS1-4, GSTS1-3, GSTS1-4, GSTD1-3, GSTD1-2, GSTD1-5, GSTD1-6, GSTD1-7, GSTD1-8, GSTD1-9, GSTD1-10, GSTD1-11, GSTT, GSTD1-12, and GSTD1-13) were significantly increased in the field KL strain ($p<0.05$) compared to the susceptible VCRU strain. GSTD1-1, GSTD1-2 and GSTS1-5 showed no significant difference in expression between the two strains ($p>0.05$). However, while comparing the expression of GST isoforms in field KL larvae and laboratory permethrin-treated strain, the same pattern was observed for all the GSTs especially for Theta and Sigma class ($p>0.05$). In Delta class, only GSTD1-1, GSTD1-9, GSTD1-11, GSTD1-12 and GSTD1-13 shows significant difference in expression between these two strain ($p<0.05$). This comparative data on GST expression in *Ae. albopictus* can be useful database to identify possible underlying mechanisms governing insecticide resistance by GSTs.

INTRODUCTION

Aedes albopictus was first identified by Skuse in 1894 (Pedigo, 2008) and it is one of the most important mosquito species in Malaysia because it is a vector for some diseases which is a threat to humankind. According to WHO (2009), *Aedes aegypti* is the primary vector of dengue and *Ae. albopictus* also acts as a secondary vector of the disease depending on its geographic location. Dengue fever was first reported in Malaysia in 1902 (Nazni *et al.*, 2009) and *Ae. albopictus* has become prevalent in urban areas; which is where *Ae. aegypti* also occurs.

Since dengue vaccines are yet to be licensed or commercialised, efforts at inhibiting this fever are directed to the primary and secondary vectors. Habitat reduction, eliminating breeding sites and use of insecticides are the proponents of these efforts. However, resistance in *Aedes* mosquitoes to several insecticide classes have been detected. Four main mechanisms have been developed by mosquitoes through which they protect themselves from toxic effects of insecticides. The mechanisms are behavioural resistance, cuticular resistance, alteration of target-sites, and enhanced detoxification (Alias and Clark, 2007; Sokhna *et al.*, 2013).

Glutathione S-tranferases (GSTs) play a crucial role in the detoxification process of a broad spectrum of noxious chemicals that have a possibility of initiating mutagenic events (Hayes and Pulford, 1995). GST-mediated detoxification process has been reported to be the cause of resistance against organophosphates, organochlorines and pyrethroids (Motoyama and Dauterman,

1975). Isoenzymes of GSTs are ubiquitously distributed in nature and are found in organisms as diverse as microbes, insects, plant, fish and mammals as a major phase II detoxification enzyme (Sheehan *et al.*, 2001). They function as a detox to a wide range of hydrophobic toxic compounds by catalysing the conjugation of glutathione to the hydrophilic centre of the toxic substances such as drugs, herbicides, insecticides and toxic endogenous substrates (Hayes and Pulford, 1995). These human-made chemicals and natural toxic compounds make up the exogenous substrates of GSTs. Metabolites produced under oxidative stress make up the endogenous substrates of GSTs. Certain GSTs carry important function in the metabolic pathway like aromatic amino acid degradation (Fernandaz-Conan and Penalva, 1998), steroid hormones (Johansson and Mannervik, 2001) and eicosanoid synthesis (Jowsey *et al.*, 2001). Hydrophobic compounds that are non-inherited substrates can also be bound by GSTs (Oakley, 2011). In additional, this reaction has been implicated the activation of one compound to a mutagenic form (Reiss and James, 1993).

Most GSTs comprises two subunits forming homodimers or heterodimers, with each subunit ranging from 17 to 28 kDa as they are cytosolic (Alias and Clark, 2007). Soluble GSTs are cytosolic GSTs that are present in the cytoplasm (Enayati *et al.*, 2006). There are at least eight different GST classes expressed in tissue cytosol. Most of these GST classes are encoded by multigene families. Alpha, Mu, Pi, Theta, Kappa, Sigma, Zeta and Omega are some of the GST gene families in which mammalian cytosolic GSTs have been divided into (Sheehan *et al.*, 2001). However, only six major of cytosolic GSTs which are Delta, Sigma, Epsilon, Omega, Theta and Zeta have been identified in insects (Ranson *et al.*, 2001). Members of these six classes have been identified in *Ae. aegypti*, *Anopheles gambiae* and *Drosophila melanogaster* (Ding *et al.*, 2003; Friedman, 2011). Based on the phylogenetic analysis, it has been reported that *Ae. aegypti* has 8 Epsilon GSTs genes, out of which only four putative orthologs (GSTE2-4 and GSTe8) have been identified, which suggests independent gene duplication (Ramsey *et al.*, 2010). In additional, *Ae. aegypti* also has 8 Delta, 1 Omega, 1 Sigma, 4 Theta and 1 Zeta GSTs genes (Fang, 2012).

Ae. albopictus is a major mosquito species and a secondary vector of dengue virus in Malaysia and one of the control method used during epidemics or impending epidemics is to spray ULV aerosols of chemical insecticides such as S-bioalletrin 0.14% w/w, permetherin 10.11% w/w and piperonyl butoxide 9.96% w/w (Chua *et al.*, 2005). However, the development of resistance towards insecticides would undoubtedly affect the effectiveness of control programmes. Pyrethroids and DDT resistance have been reported in many insect vectors. In Malaysia, resistance to DDT and pyrethroids has been reported in *Ae. albopictus* population (Chen *et al.*, 2013; Koou *et al.*, 2014). One of the major mechanisms of insecticide resistance in mosquitoes is the increase in the rate of metabolic detoxification which includes an increase in GSTs production. Although GSTs have been purified from many insect species including *Ae. albopictus* (Shukor *et al.*, 2014), purification and expression of the various GST classes in *Ae. albopictus* has not been studied. Therefore, in this study we purified, characterized and identified pure isoforms of different classes of GSTs from three different strains of the *Ae. albopictus* mosquito in order to analyze differences in GST isoforms and their expression responses after insecticides exposure. This is the first report of the characterization of GST classes in this mosquito species.

MATERIALS AND METHODS

Mosquitoes

Three (susceptible VCRU, field KL and laboratory permethrin-treated) strains of *Ae. albopictus* were used in this study. Field KL strain were sampled in an area of Flat Sri Selangor, Kuala Lumpur at 3N1'40.3788" Latitude and 101E46'0.66" Longitude. Selection of the sampling area was based on the high incidence of dengue fever cases for the past 3 years (Kuala Lumpur City Hall,

unpublished data). An established laboratory colony of *Ae. albopictus* was supplied by the Vector Control Research Unit (VCRU), USM, Pulau Pinang. These mosquitoes had been maintained without insecticide pressure for up to 200 generations. A permethrin-treated strain (second generations) was chosen because the adults *Ae. albopictus* field strain (Field KL) was collected from an active fogging area in Kuala Lumpur and over the past 5 years, one of the insecticides used in this area is permethrin (pyrethroid). Besides permethrin, from 2010 until 2015 malathion, cyphenothrin, temephos and deltamethrin insecticides have been used for Spray Chamber Thermal (Fogging) and Environment Fumigation Spray (ULV) activities in areas where incidences of dengue fever cases occurred (Kuala Lumpur City Hall, unpublished data). The collected adults were subjected for the insecticide susceptibility tests and its second generations larvae were used for the proteomic study. Larvae were used for the proteomic study because it gave more number of GST isoforms when compared to adults (data not shown). Fourth instar larvae were exposed overnight to the chemicals according to its diagnostic dosage where the concentration will cause 50% mortality (LC_{50}) (WHO, 1981). The specimens were collected and stored at -20°C until required.

Bioassay technique

Insecticide susceptibility tests were carried out using the WHO standard procedure (WHO, 1998). The WHO susceptibility test kits for adult mosquitoes (WHO tubes and accessories) and insecticide impregnated paper (4% DDT and 0.75% permethrin) were used. The bioassay was conducted using 2-3 days old, glucose non-feed female *Ae. albopictus* from all three strains where susceptible strain (VCRU strain) as a control.

For each insecticidal impregnated paper and control, three replicates of 25 adult female mosquitoes were introduced into the holding tubes and allowed to stand for 1 hour. The mortality was recorded for every 5 minutes. At the end of the required exposure period, the mosquitoes were transferred to a recovery tubes and fed with a pad of cotton wool soaked in 10% glucose solution. The holding tubes were kept for 24 hour at not more than 30°C. The mortality was recorded after 24 hours and plotted the against exposure time to obtain (Lethal Time) LT values by using a probit analysis in SPSS software. The lethal time at 50% mortality (LT_{50}) was obtained and the resistance ratio was calculated. If mortality between 5% and 20% was observed in the controls, the percentage mortality was recalculated using Abbott Formula (Abbot, 1925).

Sample preparation

In a standard procedure, 1g of fourth instar larvae was homogenised in 5 mL of homogenising buffer containing 25 mM Sodium Phosphate Buffer, pH 7.4 (buffer A), 1.0 mM EDTA, 0.1 mM DTT, 0.1 mM PTU and 0.5 ml protein inhibitor cocktail (Sigma). A clear crude extract was prepared by centrifugation of the homogenate at 100,000 x g for 45 minutes. The preparation was performed at all times at 4°C. The clear supernatant was designated as the crude enzyme and used for further activity test and purification procedure.

Affinity chromatography

The clear crude extraction was applied to a Glutathione Sepharose™ High Performance columns (GSTrap™ HP), equilibrated with buffer A to remove potentially interfering inhibitor pigment (Pal *et al.*, 2012). The flow rate was 0.3 ml/min. When the sample had been applied, the column was first washed with buffer A to remove non-specifically bound protein designated as void. Before eluting, the column was re-equilibrated with buffer A. The bound GSTs were eluted using 100% gradient of 10 mM glutathione, pH 7.4 (GSH, Sigma). Two peaks of GST activity were recovered from this column, designated peak 1 (unbound fraction) and peak 2 (affinity elute fraction). The fractions were assayed with 1-Chloro-2,4-dinitrobenzene, and those with activity were pooled. The pooled bound fractions were then concentrated using 20 ml concentrator (Vivaspin 20: 10 000 WMCO) by centrifuging at 6000 rpm for 20-30 minutes depending on the volume.

Enzymes assay and protein determination

All assays were performed in a Jasco V630 spectrophotometer. The GST activities towards the model substrates were determined as described by Habig *et al.* (1974). Conjugating activities with 1-Chloro-2,4-dinitrobenzene were determined at pH 9.0 and pH 7.5.

Enzyme activity was expressed as µmol/min at 25°C and the specific activity as µmol/min/mg protein. Protein concentration was determined using the Bradford protein assay with bovine serum albumin as a standard (Bradford, 1976).

Two-dimensional gel electrophoresis (2 DE gel)

Samples for first dimension separation were prepared by mixing with 8 M urea, 2% CHAPS (Molecular Biology grade), 0.15% DTT, 2% ampholytes, 30 mM thiourea and traces of bromophenol blue and were applied to a 7 cm, nonlinear Immobiline™ DryStrip pH 3-10 (GE Healthcare) for rehydration. The first dimension Isoelectric focusing was conducted using Multiphore™ II electrophoresis system at 20°C. Subsequently, the strips were equilibrated in an equilibration buffer (6 M Urea, 75 mM Tris-HCl, pH 8.8, 2% (w/v) SDS and ddH_2O) containing DTT and iodoacetamide for 30 min each. The equilibrated strips were loaded on the 12% SDS PAGE gel for second dimension electrophoresis at 120 V until the dye reached the end of the gel. Protein spots in analytic gels were visualised by Coomassie staining solution (5% (w/v) Coomassie Brilliant Blue, 85% H_3PO_4, ammonium sulphate). The gels were then scanned and analysed using Molecular Dynamics Personal Densitometer and PDquest software.

Image 2 DE gel and statistical analysis

Coomassie Blue stained gels were scanned (as previously described) and the tif.images were generated using PdQuest software to obtain the density of every spot visualized on the gel. Quantitative different were sought in spots that were present in all gels and the mean of protein content of scanned spots were compared. The individual protein spot was quantified by calculated the density distribution generated by the PdQuest software to protein amount.

All experiments were performed in triplicate (n=3). The data were presented as mean ± standard deviation (S.D.) and were statistically analysed with the SPSS software version 19.0. The LT50 values of WHO standard assay were obtained from Probit analysis test from this software. For differential study, the values were compared and the significant ($p<0.05$) difference were tested with T-test from the SPSS software version 19.0.

RESULTS

The field KL strain, permethrin-treated laboratory strain and susceptible VCRU strain were exposed to 4% DDT and 0.75% permethrin for different time periods. After recovering for 24 hours, the mortality was recorded and analysed using probit analysis from the SPSS software to obtain the LT_{50} values. The LT_{50} value for 4% DDT were 26.10±0.59 min, 42.70±0.19 min and 27.24±0.14 min in VCRU susceptible strain, KL strain and permethrin-treated strain, respectively with the resistance ratio of 1.63±0.02 for the KL strain and 1.04±0.02 for the permethrin-treated strain. The LT_{50} values for 0.75% permethrin were 18.56±0.23 min, 65.55±0.50 min and 66.94±0.80 min in VCRU susceptible strain, KL strain and permethrin-treated strain respectively with the resistance ratio of 3.53±0.04 for KL strain and 3.61±0.04 for permethrin-treated strain (Table 1).

The prepared crude extracts (100,000 x g supernatant) of *Ae. albopictus* larvae from the three different strains contained considerable amount of GST activity. The results are shown in Table 2 where the crude protein from the susceptible VCRU strain showed the highest specific activity (1.266±0.001 µmol/min/mg) compared to other strains. However, the crude protein from field KL and laboratory permethrin-treated strains show almost similar specific activities which are 0.992±0.047 and 0.978±0.108 µmol/min/mg.

Table 1. LT_{50} value of three different strains of *Aedes albopictus* mosquito when exposed to 4% DDT and 0.75% permethrin

Samples (strains)	4% DDT		0.75% permethrin	
	LT_{50} value (min)	Resistance ratio	LT_{50} value (min)	Resistance ratio
Susceptible VCRU	26.10±0.59	1	18.56±0.23	1
Kuala Lumpur field	42.70±0.19	1.63±0.02	65.55±0.50	3.53±0.04
Lab permethrin-treated	27.24±0.14	1.04±0.02	66.94±0.80	3.61±0.04

The values represent mean ± SD of triplicate, independent experiments.

Table 2. Purification of GSTs from three different strains of *Aedes albopictus* mosquito

Strains	Steps of Purification	Total protein (mg)	Total activity (µmol/min)	Specific activity (µmol/min/mg)	Purification Fold	Yield (%)
Susceptible VCRU strain	Crude	2.073±0.014	2.623±0.002	1.266±0.001	1	100
	Unbound	0.846±0.021	1.889±0.019	2.233±0.023	1.7	72
	Affinity elute	0.242±0.005	1.597±0.009	6.586±0.039	5.2	61
Field KL strain	Crude	3.558±0.078	3.527±0.094	0.992±0.047	1	100
	Unbound	1.020±0.055	1.946±0.024	1.913±0.108	1.9	55
	Affinity elute	0.351±0.014	1.619±0.012	4.620±0.166	4.4	45
Permethrin-treated	Crude	3.579±0.149	3.490±0.247	0.978±0.108	1	100
	Unbound	1.023±0.052	1.959±0.022	1.919±0.079	2.0	56
	Affinity elute	0.345±0.012	1.608±0.021	4.667±0.113	4.8	46

GSTs (crude) of all samples were purified using GSTrap™ HP column. The substrate used is CDNB. The values represent mean ± SD of triplicate, independent experiments.

To further characterize GST activity, the crude extractions of these larvae were separately applied onto a Glutathione Sepharose™ High Performance column (GSTrap™ HP) at 0.5 ml for every minute and relatively pure enzymes were obtained. In respect of 1-Chloro-2,4-dinitrobenzene the percentage yields of GSTrap™ HP isolated from susceptible VCRU, field KL and laboratory permethrin-treated strains were 61%, 45% and 46% respectively (Table 2). Despite the lowest yield, the purified field KL and laboratory permethrin-treated strains GSTs enzyme showed high protein amounts which are 0.351±0.014 mg and 0.345±0.012 mg. With 1-Chloro-2,4-dinitrobenzene substrate conjugation, the purified GSTs of susceptible VCRU strain shows higher specific activity which is 6.586±0.039 µmol/min/mg. The purified GSTs of the field KL and laboratory permethrin-treated strains showed almost similar specific activities which were 4.620±0.166 and 4.667±0.113 µmol/min/mg respectively.

Table 3 shows the comparison of protein amount (µg) of the GST isoforms from the three different strains (susceptible VCRU, field KL and laboratory permethrin-treated strains). The T-Test (paired sample test) was applied to examine the significant difference between the protein content of the isoforms in susceptible VCRU and Field KL strains and also between the laboratory permethrin-treated and the field KL strains. The quantitative analysis shows significant difference ($p<0.05$) in expression of the sixteen isoforms (GSTS1-1, GSTS1-2, GSTS1-3, GSTS1-4, GSTD1-3, GSTD1-4, GSTD1-5, GSTD1-6, GSTD1-7, GSTD1-8, GSTD1-9, GSTD1-10, GSTD1-11, GSTT, GSTD1-12, and

Table 3. Protein amount (µg) of the GST isoforms *Aedes albopictus* from three different strains

Spots	Proposed Identification	Fold change KL/ VCRU	VCRU strain (µg)	KL strain (µg)	Permethrin-treated strain (µg)	Fold change Permethrin -treated/ KL
1	GSTS1-1	**1.7**	2.38 ± 0.15^{a}	3.86 ± 0.26^{b}	4.74 ± 0.19^{b}	1.1
2	GSTS1-2	**1.9**	2.38 ± 0.03^{c}	4.57 ± 0.67^{d}	4.68 ± 0.06^{d}	NC
3	GSTS1-3	**1.4**	6.13 ± 0.22^{e}	8.27 ± 0.59^{f}	8.65 ± 0.14^{f}	NC
4	GSTS1-4	**2.0**	2.68 ± 0.04^{g}	5.29 ± 0.18^{h}	4.65 ± 0.46^{h}	1.1
5	GSTD1-1	NC	3.64 ± 0.06^{i}	3.13 ± 0.02^{i}	6.48 ± 0.49^{j}	**2.0**
6	GSTS1-5	NC	1.86 ± 0.04^{k}	1.95 ± 0.09^{k}	1.87 ± 0.21^{k}	NC
7	GSTD1-2	1.2	6.43 ± 0.15^{l}	7.80 ± 0.21^{l}	8.61 ± 0.13^{l}	1.1
8	GSTD1-3	**2.6**	2.47 ± 0.05^{m}	6.51 ± 0.26^{n}	5.71 ± 0.11^{n}	1.1
9	GSTD1-4	**1.5**	1.31 ± 0.04^{o}	2.03 ± 0.34^{p}	2.52 ± 0.12^{p}	1.2
10	GSTD1-5	**2.1**	2.79 ± 0.04^{q}	5.92 ± 0.60^{r}	5.19 ± 0.30^{r}	1.1
11	GSTD1-6	**1.3**	6.18 ± 0.12^{s}	8.24 ± 0.20^{t}	8.57 ± 0.15^{t}	NC
12	GSTD1-7	**2.1**	5.83 ± 0.50^{u}	2.81 ± 0.45^{v}	2.28 ± 0.06^{v}	1.2
13	GSTD1-8	**1.9**	2.39 ± 0.03^{w}	4.48 ± 0.14^{x}	5.17 ± 0.36^{x}	1.2
14	GSTD1-9	**2.3**	2.17 ± 0.04^{y}	5.07 ± 0.72^{z}	3.65 ± 0.39^{aa}	**1.4**
15	GSTD1-10	**1.4**	2.69 ± 0.02^{ab}	3.76 ± 0.23^{ac}	3.65 ± 0.18^{ac}	NC
16	GSTD1-11	**2.3**	1.88 ± 0.03^{ad}	4.30 ± 0.20^{ae}	3.21 ± 0.35^{af}	**1.3**
17	GSTT	**2.0**	1.88 ± 0.04^{ag}	3.72 ± 0.36^{ah}	3.52 ± 0.28^{ah}	NC
18	GSTD1-12	**3.1**	0.87 ± 0.05^{ai}	2.71 ± 0.34^{aj}	3.52 ± 0.28^{ak}	**1.3**
19	GSTD1-13	**2.0**	1.74 ± 0.12^{al}	3.54 ± 0.12^{am}	2.35 ± 0.15^{an}	**1.5**

GSTs were purified using GSTrap™ HP column. The values represent mean ± SD of triplicate, independent experiments. Proposed identification of nineteen isofroms were successfully identified as three classes of GST (NCBInr) which are Sigma, Delta and Theta. All spots are designated as GSTS1-5, GSTD1-13 and GSTT accordingly to the ascending order of their pI values spotted in 2DE gel. The protein content was calculated based on the spot density detected from PDquest software. Bold numbers indicate the difference between control and treated samples are significant at $P<0.05$. NC indicates "No change". Protein amount with same letters show no significant difference at $p = 0.05$.

GSTD1-13) between the susceptible VCRU and the Field KL strains. Isoforms GSTD1-1, GSTD1-2 and GSTS1-5 show similar protein amounts between these two strains.

There was no significant difference ($p>0.05$) in protein amount for almost all the GST isoforms between the laboratory permethrin-treated and the field KL strains except for GSTD1-1, GSTD1-9, GST1-11, GST1-12 and GST1-13 ($p<0.05$). It illustrates similar expression pattern for Sigma and Theta GST isoforms of the *Aedes* larvae between these two strains (Figure 1).

DISCUSSIONS

The purification and expression of *Ae. albopictus* GSTs has previously been conducted by Shukor *et al.* (2014) but it was not as extensive as this study. This is the first report of GST isoforms from different classes that have been purified to homegenity isolated from *Ae. albopictus*. Most of the works on GSTs, especially in insects have used an affinity matrix for selective isolation, in order to minimize non-GST contamination (Alias and Clark, 2007). This provides better visualization on the 1D SDS-PAGE. Most insects and mosquitoes GST isoforms have similar molecular weight (MW) based on bioinformatics analysis which in the range of 17 to 28 kDa (Alias and Clark, 2007; Lumjuan *et al.*, 2007). Analysis with only SDS-PAGE would not resolve these GSTs effectively. By using a combination of chromatography and 2DE gel analysis, the present study successfully isolated, identified and characterized *Ae. albopictus* GSTs. This study could serve as an initial step in detailed elucidation of the identification and classification of *Ae. albopictus* GSTs. Identification especially on the inducible GSTs is an important way to find the candidate

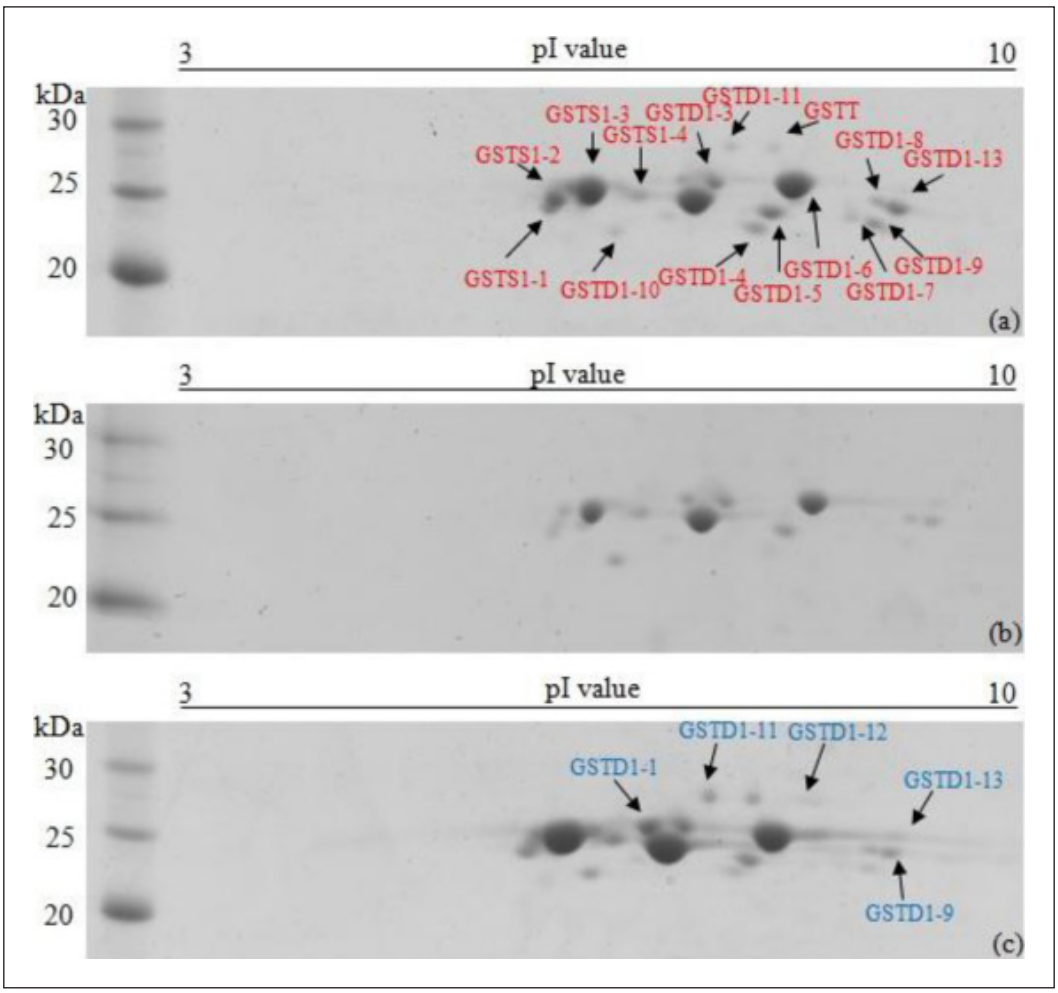

Figure 1. 2DE gels comparison of GSTrap™ HP isolated GSTs of (a) field KL strain, (b) susceptible VCRU strain and (c) permethrin-treated strain.
There is a significant difference ($p<0.05$) between field KL and susceptible VCRU strains for each red labelled isoforms and a significant difference ($p<0.05$) between field KL and permethrin-treated strains for each blue labelled isoform. The same volume of concentrated sample was focused on IPG strip pH 3-10 NL. Gels were stained with coomassie blue staining method. Bencmark marker (Invitrogen) was used to estimate the molecular weight.

For Black and White figure
There is a significant difference ($p<0.05$) between field KL and susceptible VCRU strains for each labelled isoforms in (a) and a significant difference ($p<0.05$) between field KL and permethrin-treated strains for each labelled isoform in (c). The same volume of concentrated sample was focused on IPG strip pH 3-10 NL. Gels were stained with coomassie blue staining method. Bencmark marker (Invitrogen) was used to estimate the molecular weight.

GSTs conferring in the insecticide resistance. More studies have been focused on the identification and characterization of insects GST which involved peptide mass fingerprinting analysis, DNA microarray technology and transcriptome sequencing (Ranson *et al.*, 2001; Alias and Clark, 2007). The MALDI-TOF and DNA microarray was mainly used in the model organisms, which its genome have been sequenced. As for non-sequence GSTs (*Ae. albopictus*), the development of sequencing technology (transcriptome sequencing) is a suitable alternative for further analysis to its whole

genome sequencing and can be used to characterize the resistant gene at the level of transcription (Fang, 2012).

GSTs are present in almost all eukaryotes where they occur in multiple isoenzymic forms, constituting a significant intracellular mechanism of detoxification (Chen *et al.*, 2012). Many preliminary studies of insect GSTs reveal the existence of multiple forms. These include house fly, *Musca domestica* (Motoyama and Dauterman, 1978; Franciosa and Berge, 1995; Nay *et al.*, 1999), honeybee, *Apis cerana cerana* (Yan *et al.*, 2012; Yu *et al.*, 2012) and fruit fly, *D. melanogaster* (Prapanthadara, 1993; Singh *et al.*, 2000; Alias and Clark, 2007; Low *et al.*, 2010). There are at least twenty-six cytosolic GST forms in the *Ae. aegypti* with eight, one and four are from Delta, Sigma and Theta classes respectively (Grant and Matsumura, 1988; Grant *et al.*, 1991; Lumjuan *et al.*, 2005; Azael *et al.*, 2009; Friedman, 2011; Fang, 2012) and thirty-eight forms in *Culex quinquefasciatus* with seventeen, two and six cytosolic GSTs are from Delta, Sigma and Theta classes respectively (Kasai *et al.*, 2009; Friedman, 2011; Reddy *et al.*, 2011; Fang, 2012). Fifteen forms of GST from Delta, Sigma and Theta classes have also been partially purified in *An. gambiae* (Prapanthadara, 1993; Friedman, 2011; Fang, 2012). Therefore, this study shows that several classes of GST as already reported in other mosquitoes also exist in *Ae. albopictus* (Delta, Sigma and Theta).

Resistance to DDT and pyrethroids has been reported in insects (Chadwick *et al.*, 1977; Plapp and Hoyer, 1968). The two main mechanisms involved are alteration in the sodium channel and increased production of GSTs (Kostaropoulos *et al.*, 2001). In Malaysia, resistance to DDT and pyrethroid has been reported in *Ae. albopictus* population (Nazni *et al.*, 2009; Wan-Norafikah *et al.*, 2010).

As compared with the susceptible VCRU strain, the field KL strain was slightly resistant to DDT with low resistance level at 1.63±0.02 and resistant to permethrin at 3.53±0.04, however the laboratory permethrin-treated strain was only resistant to permethin. Elevated GSTs activity implicated in conferring resistance to DDT and pyrethroid insecticides in insects (Grant and Mutsumura, 1988; Prapanthdara and Ketterman, 1993; Vontas *et al.*, 2001) was detected in the field KL strain relative to the susceptible VCRU strain which might involve the mechanism of enhanced detoxification. The use of permethrin in the sampling area as a control strategy may explain the resistance observed in the field KL strain. The use of DDT for mosquito control had previously been stopped due to its ban in 1972. However, it has been reintroduced for use by the WHO since 2006 for controlling malaria diseases (WHO, 2011). According to Penilla *et al.* (2006), it is possible that persistent DDT contamination in the environment maintains a selection pressure on mosquito population. DDT and DDE persist in the environment, especially in soils in areas treated with DDT in the past. Because DDT and DDE do not degrade quickly in the environment, the amounts that may be left behind from applications that ceased three decades ago may be significant (Walker and Powell, 2004). According Walker and Powell (2004), studies have shown that DDT has a half-life in the soil of between 2 and 15 years, depending upon moisture and temperature condition. He concluded that if one pound (0.454 kg) of DDT were applied to the soil in 1972, between a negligible amount and 23% of the original amount would be left in 2003. Therefore insecticide use for personal protection, controlling crop and pests from time to time has often been suggested as additional selective pressures favouring insecticides resistance. The development of resistance in the field strain would negatively affect the control of *Ae. albopictus* using permethrin in the sampled area.

The possible interaction between GST and resistance in *Ae. albopictus* towards DDT and permethrin showed that the field KL and laboratory permethrin-treated strains showed almost similar specific activity. This suggests that GSTs of the laboratory permethrin-treated and the field KL strain show similar activity pattern and conjugation towards permethrin.

In the advanced proteomic study in this present work, the interactions of GST isoforms for the laboratory permethrin-treated were not the same as in the field KL strain. Almost all the GST isoforms of the field KL strain were overexpressed ($p<0.05$) on 2DE gel relative to the susceptible VCRU strain except for the isoforms GSTD1-1, GSTD1-2, and GSTS1-5. Comparing with the susceptible VCRU strain, GSTD1-1, GSTD1-9, GSTD1-11, GSTD1-12 and GSTD1-13 showed significant differences and increased GST expression patterns ($p<0.05$) between permethrin-treated and field KL strain. These suggest that specific GST isoenzymes may play roles in cellular antioxidant defences either in the lab or field environment conditions. In Malaysia pyrethriods are widely used for dengue vector control, the enhanced expression of some of the GSTs isoforms (Delta, Sigma and Theta GSTS) as in this study might affect the control programme. According to Chen *et al.* (2003), Delta GSTs are associated with the exposure to the organochlorine and pyrethriod insecticide, where agGSTd-6 from *An. gambiae* metabolizes DDT and is inhibited by number of pyrethriod insecticides. In another study, AccGSTS1 identified in *Apis cerana cerana* showed up regulation in response to all insecticide treatments conditions which suggested this specific Sigma class isoforms is a crucial antioxidant enzyme involved in cellular antioxidant defences and honey bee survival (Yan *et al.*, 2012). Furthermore, reports correlating high levels of metabolic enzymes such as GST with high resistance to pyrethroids exist for other mosquito species such as *Ae. aegypti* (Grant and Matsumura, 1988) and *An. arabiensis* (Nardini *et al.*, 2013). It has also been detected that another group of metabolic enzymes, cytochrome P450 was over-expressed in surviving *Ae. albopictus* challenged with domestic pyrethroid insecticides (Avicor *et al.*, 2014). Hence the high activities of metabolic enzymes such as GSTs expressed by the *Ae. albopictus* mosquito in this study may help it to overcome chemical challenges.

CONCLUSION

This study extensively investigated GST isoforms from different classes in the *Ae. albopictus* mosquito. Different GST's expression response was observed from three different strains of *Ae. albopictus* larvae. Our results support the idea that there is a response to pyrethroid insecticides intoxication in *Ae. albopictus* and that the GST's Sigma, Delta and Theta class play role in this. This differential study presented some basic proteomic information of GST from different strains of *Ae. albopictus*, and these information are worthy of closer scrutiny in future studies on the exact role and evolutionary important in detoxification of the various GST isoenzymes.

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