CHAPTER TWO

MATERIALS AND METHODS

2.1 Samples

The collection of blood samples from healthy blood donors and the genomic DNA extraction were done and documented by Nadarajan & Shanmugam (2005). The blood donors were composed of different ethnic groups of Malaysian population including Malays, Chinese and Indians in addition to ethnic groups of East Malaysians. The distribution of the donors were 649 Malays, 641 Chinese, 371 Indians, 31 East Malaysians and 31 foreigners which are from different countries, for example, Indonesia, Thailand, Pakistan, Bangladesh and Brunei.

2.2 Methods

2.2.1 Methods Done Previously

2.2.1.1 Serological Analysis and Real-time PCR

For the samples that were included in the study, serological tests and Real-time PCR were done previously as described by Nadarajan & Shanmugam (2005). The serological tests were done on 1723 blood samples that were collected from healthy blood donors to detect Mi^a phenotype by using anti-Mi^a monoclonal antibody (GAMA210). Out of the 1723 samples, 79 were anti-Mi^a positive. These 79 samples were analyzed by Real-time PCR after the extraction of the genomic DNA to detect *GYP* (*B-A-B*) gene conversion events by using forward primers complementary to the pseudo-exon region of *GYB* and reverse primers specific for *GYA*.

2.2.2 Polymerase Chain Reaction – Sequence Specific Primer (PCR-SSP)

Seventy seven out of the 79 archived DNA samples were included in this study. The samples consist of different ethnic groups as follows: 34 Malays, 34 Chinese, 6 Indians and 3 foreigners.

2.2.2.1 Primers used in PCR - SSP

Specific designed primers for detection of *GYPs* were used as described by Palacajornsuk *et al.* (2007) (Table 2.1). F2 located in intron 2 and extends four nucleotides into exon 3 and Rccgg located in exon 3 and extends 28 nucleotides into intron 3, which amplified fragments of 148 bp (GP.Mur, GP.Hop and GP.Bun) and 151 bp (GP.HF and GP.Hut), respectively. In addition, HGH (Human growth hormone) was used in this study as an internal control and the size of the product is 434 bp.

Table 2.1: Primers used in the study (Palacajornsuk *et al.*, 2007).

Primer name	Location	Sequences	Specific for
F2 Rccgg	nt 817–847 nt 964–934	5′-ccc ttt ctc aac ttc tct tat atg cag ATA A-3’ 5′-gag caa cta ttt aaa act aag aac ata cCG G-3’	GP.Hut, GP.Mur, GP.Hop, GP.Bun and GP.HF
HGH5580 F	nt 5559-5581	5′-TGC CTT CCC AAC CAT TCC CTT A-3′	HGH
HGH5967 R	nt 5992-5967	5′-cca ctc acG GAT TTC TGT TGT GTT T-3′	

2.2.2.2 The PCR-SSP Technique

Preparation of PCR reagents, sample preparation, PCR setup and analysis of PCR products were performed under aseptic conditions to avoid contamination and false positive results.

Some examples of the precautions that were used to avoid sample contamination are as follows: (1) Separate rooms for reagent and sample preparation; amplification and visualization of PCR products. (2) Dedicated micropipettes with sterile, disposable tips. (3) Aseptic technique including the wearing of gloves. (4) Following strict laboratory rules, for instance, samples and controls preparation should be done in designated benches. (5) Using suitable negative controls.

In this study, negative controls were included in each run of PCR that contained all PCR reagents except DNA template which was replaced by RNase-free water to monitor contamination. Negative control samples which referred as NTC (non template control) should not show successful amplification.

The PCR-SSP was used in the study as monoplex PCR, the PCR Mix for 1 reaction of the total PCR volume of 25 µl was prepared by adding specific volumes of PCR reagents; 12.5 µl of PCR Master Mix, 1 µl of each forward and reverse Mi Primers and the total volume was adjusted to 25 µl by adding 8.5 µl of RNase-free water plus 2 µl of archived DNA sample. Similarly, PCR reactions mixtures were prepared for different archived genomic DNA samples and aliquot into 200 µl PCR tubes. The internal controls were prepared by using HGH Primers instead of Mi Primers; the prepared PCR mixture either inserted directly in a PCR machine (Eppendrof Mastercycler® Gradient) for amplification.

In PCR, approximately 100 ng of genomic DNA per reaction was amplified by HotStar *Taq* DNA polymerase in a reaction mixture containing $MgCl_2$, PCR buffer, deoxynucleosides triphosphate (QIAGEN Inc., Valencia, CA, USA), forward and reverse of each set of Mi primers. The PCR was performed for 35 cycles in a thermal cycler (Eppendorf AG, Hamburg, Germany) to activate DNA polymerase enzyme as follows: started by initial denaturation at 95°C for 15 min, followed by 35 cycle of (1) denaturation at 94°C for 20 s (2) annealing at 65°C for 20 s (3) extension at 72°C for 20 s followed by final extension at 72°C for 10 min (Table 2.2). These temperatures that used in the PCR-SSP were based on published article by Palacajornsuk *et al.*, 2007.

Table 2.2: PCR Conditions for amplification of the *GYP* genes.

No.	PCR Step	Temperature	Time
1	Initial Denaturation	95 °C	15 min
2	35 cycle of :		
	A- Denaturation	94 °C	20 s
	B- Annealing	65 °C	20 s
	C- Extension	72 °C	20 s
3	Final Extension	72 ˚C	10 min

2.3 Analysis of PCR Products

2.3.1 Preparation of 1.7% Agarose Gel

1.7 % agarose gel was prepared by mixing of 1.36 g of agarose powder (Invitrogen) with 80 ml of 10 X Tris-borate-EDTA (TBE) electrophoresis buffer to get the desired concentration. The agarose was heated in a microwave oven for about 3 min until it was completely melted. The molten gel was cooled down to approximately 60˚C. The gel was stained by adding 1 µl of 10 mg/ml of Ethidium Bromide as a fluorescent dye to facilitate visualization of DNA after electrophoresis. The molten gel was poured into a clean gel casting tray with inserted sample combs. Prior to pouring of gel, the teeth of the comb were cleaned and caution was taken to ensure there was no bubble formation in the gel on the tray; otherwise, the bubbles were removed by using a sterile micropipette tip. The gel was

allowed to solidify at RT for about 15-20 min before use, to allow the formation of sample wells in the gel.

2.3.2 Agarose Gel Electrophoresis

After the gel solidified, the comb was removed carefully to avoid ripping the bottom of the wells. The gel, while still in its plastic tray, was inserted horizontally into the electrophoresis chamber and covered with sufficient amount of 10X TBE electrophoresis buffer of pH 8.0 subsequently until it was completely surrounded. Samples containing 10 µl of PCR product mixed with 2 µl of 5 X Blue juice loading dye (Invitrogen) were then loaded into specific sample wells. At the same time, the NTC sample was loaded in the first well, whereas 2 µl of a 100-bp DNA Ladder Plus (Invitrogen) was loaded as a molecular size marker to estimate the PCR product size.

After the lid and power leads were placed on the apparatus correctly, electrophoresis was conducted at RT with a constant voltage of 120 V for 30 min. After the agarose gel was electrophoresed, it was visualized by using a transilluminator device and results were documented via photography. Precautions, for example, wearing of suitable gloves were taken with handling of the Ethidium bromide solution and stained gel due to its mutagenic compounds.