CHAPTER ONE

INTRODUCTION

1.1 Blood Group Antigens

In the human blood, there are different blood group systems. The ABO blood group system was the first blood group to be discovered by Landsteiner in 1900. Following that, there has been an increase in the understanding of human blood group antigens. As a result, more than 250 red blood cell (RBCs) antigens have been identified and arranged into 29 major blood systems by Working Party of the International Society of Blood Transfusion (ISBT) (Daniels *et al*., 1995; Hillyer *et al*., 2001; Lewis *et al.,* 2001). Some examples of these blood group systems are: MNS, P, Rh, Kell, Kidd, Lutheran, and Duffy system. Moreover, other RBCs antigens that are not categorized into major systems have been classified into collections. Some antigens have no known alleles and cannot be placed in a blood group system or collection and have been classified into a series: (1) the 700 series of low-prevalence (2) the 901 series of high-prevalence antigens (Daniels *et al*., 1995; Reid *et al*., 2000; Hillyer *et al*., 2001; Lewis *et al.,* 2001; Reid & Lomas- Francis, 2004).

Blood group antigens are surface markers that are situated on the surface of the RBC membrane. Based on the structure, they are carbohydrate or protein attached to lipid or protein (Reid *et al*., 2000; Lewis *et al.,* 2001; Reid & Lomas, 2004). The clinical importance of blood group antigens is the immune response that occurs with

some individuals who have received blood with antigens lacking on their RBCs. Therefore, the significance of these antigens can be discerned in varied clinical situations such as maternofetal blood group incompatibility, autoimmune hemolytic anemia and organ transplantation in addition to other hemolytic transfusion reactions (Reid *et al*., 2000).

1.2 The MNS Blood Group System

In 1927, Landsteiner and Levine discovered a second blood group system, which is known as the MNS blood group system. The discovery was a result of immunizing rabbits with human RBCs in the hope that polymorphic differences on the RBC surface could be distinguished (Erskine & Wladyslaw, 1978; Reid & Lomas, 2004; Palacajornsuk, 2006). However, The MNS blood group system is considered as a highly polymorphic blood group system and contains many low-incidence antigens that are closely linked (Giles, 1982; Blumenfeld & Huang, 1997; Palacajornsuk *et al.*, 2007).

1.2.1 Antigens of MNS System

After the identification of the M or N and S or s antigens, more than 40 distinct antigens associated with the MNS blood group system have been detected. A large proportion of these antigens are low incidence. Some examples of the MNS system antigens are: U, He, DANE, TSEN and Mi^a antigens (Lewis *et al*., 1990; Blumenfeld & Huang, 1997; Palacajornsuk, 2006). The incidence of different phenotypes of the MNS blood system is diverse and varies throughout the world depending on the ethnic groups and geographical locations (Blumenfeld & Huang, 1997).

1.2.2 The Miltenberger (Mi) Subsystem

1.2.2.1 Definition

The Miltenberger (Mi) subsystem represents a group of phenotypes for RBCs that carry low incidence antigens associated with the MNS blood group system. It was first defined by Cleghorn in 1966 when RBCs reacted with serum from Mrs Miltenberger (anti-Mi^a); hence, it is called the Miltenberger subsystem (Chandanayingyong & Pejrachandra, 1975; Lin *et al*., 1996; Chen *et al*., 2001; Reid & Lomas- Francis, 2004).

1.2.2.2 The (Mi) Subsystem Antigens

Initially, the (Mi) subsystem consisted of four phenotypes. The RBCs reactive with the anti-Mi^a serum were classified into four classes based on their different reactions with four type of sera called Verweyst (Vw), Miltenberger (Mi^a), Murrel (Mur), and Hill (Hil). Subsequently, the (Mi) subsystem extended as RBCs were found to give different patterns of reactions with defined antibodies. Thus, the Mi subsystem developed to 11 groups of Miltenberger phenotypes namely; from Mi.I to Mi.XI (Chen *et al*., 2001; Palacajornsuk, 2006). However, a terminology for the (Mi) subsystem antigens that depends on the glycophorin (GP) as proposed by Tippet *et al.* (1992) is nowadays usually used (Lin *et al*., 1996; Reid & Lomas- Francis, 2004) (Table 1.1).

Table 1.1: Terminology of the Miltenberger (Mi) subsystem, which have been named from Mi.I to Mi.IX in addition to the terms based on the glycophorins, adapted from Reid & Lomas- Francis, (2004).

Terminology	
Mi.	**GP.**
Mi.I	GP.Vw
Mi.II	GP.Hut
Mi.III	GP.Mur
Mi.IV	GP.Hop
Mi.V	GP.Hil
Mi.VI	GP.Bun
Mi.VII	GP.Nop
Mi.VIII	GP.Joh
Mi.IX	GP.Dane
Mi.X	GP.HF
Mi.XI	GP.JL

GP.: glycophorin

1.3 Molecular Basis of MNS System Antigens

1.3.1 Glycophorins Genes

Glycophorins are a group of sialoglycoproteins which are present on the membrane of human erythrocytes. The Glycophorin A (GPA) and Glycophorin B (GPB) are the two major sialoglycoproteins. The GPA gene *(GYPA)* and GPB gene *(GYPB)* are located on the long arm of chromosome 4. Moreover, a third glycophorin gene**,** denominated *GYPE* lies adjacent to *GYPB* which participates in the gene rearrangements resulting in variant alleles (Huang & Blumenfeld, 1991(a); Reid & Lomas- Francis, 2004; Daniels *et al*., 2004; Palacajornsuk, 2006). In addition, *GYPB* and *GYPE* share considerable homology with *GYPA* and arise by gene duplication from a single, ancestral *GYPA* gene (Kudo *et al*., 1990; Huang & Blumenfeld, 1991; Storry *et al.,* 2000*;* Palacajornsuk, 2006).

Kudo & Fukuda (1989) compared the genomic structure of GPA and GPB by analyzing the DNA clones and they argued that the *GYPA* has 7 exons, which is also mentioned by Reid & Lomas- Francis (2004) and Palacajornsuk (2006). The exon arrangement of *GYPA* and *GYPB* is similar. Nevertheless, in *GYPB*, there is a splice site mutation that has inactivated the splicing consensus sequence at the 5′ end of intron 3. Accordingly, this exon is known as a pseudoexon (non-coding exon) which is spliced out and exon 2 joins to exon 4 consequently. Therefore, *GYPB* has 5 exons (exon 3 in *GYPB* is a pseudoexon) (Kudo & Fukuda, 1989; Reid & Lomas-Francis, 2004; Palacajornsuk, 2006). Similarly, *GYPE* has 4 exons (Exon 3 and 4 in *GYPE* are pseudoexons (Fig. 1.1) (Blumenfeld & Huang, 1997; Palacajornsuk, 2006).

Exon 1 and part of the 5′ end of exon 2 in each of the GP genes encode the leader sequence for the corresponding glycophorin, while exon 2 to 4 encode the extracellular domains (Blumenfeld & Huang, 1997; Palacajornsuk, 2006), exon 5 encodes the transmembrane domains of each glycophorin, and exon 6 and part of the 5′ end of exon 7 of *GYPA* encode the cytoplasmic domain of GPA (Fig. 1.1) (Palacajornsuk, 2006).

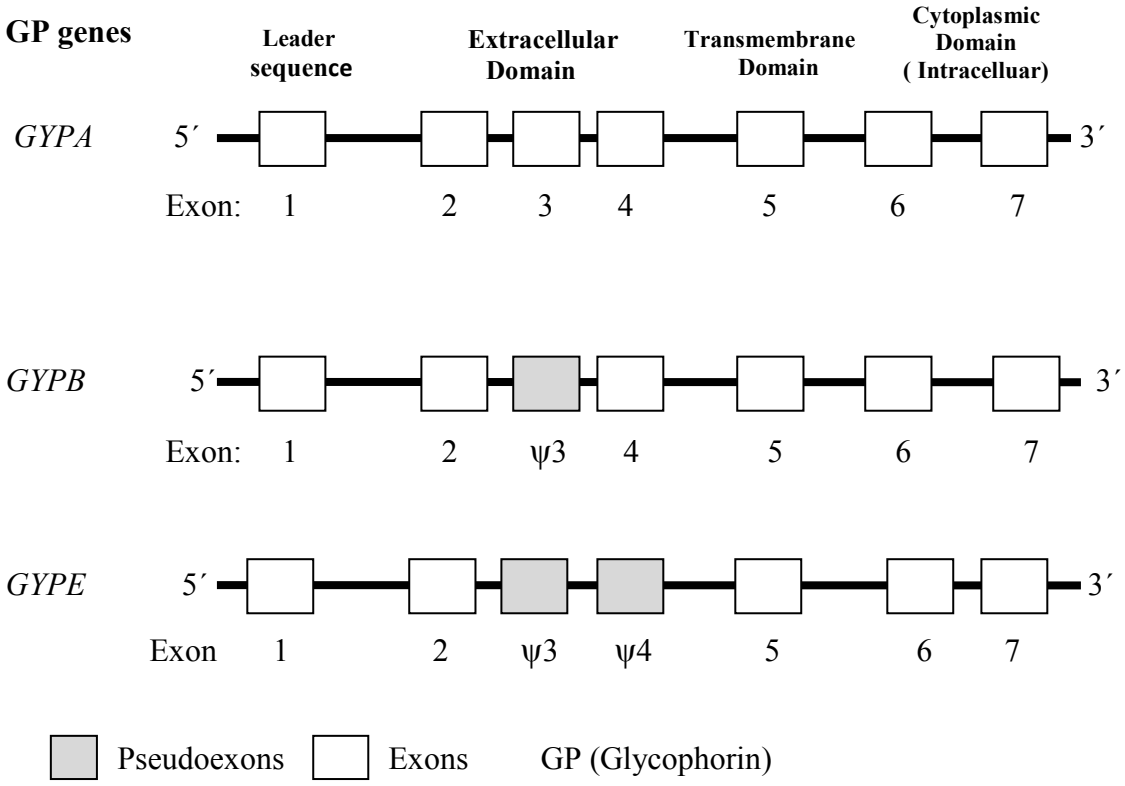


Fig. 1.1 Genomic organization of *GYPA*, *GYPB* and *GYPE*. (Adapted from Palacajornsuk, 2006).

The isolation of cDNA and genomic clones of GPA and GPB results in clarification of their gene structure and organization that led to understanding of the high homology between the primary sequences of GPA and GPB genes (Huang & Blumenfeld, 1991-a; Kudo *et al*., 1990). However, *GYPA* and *GYPB* share about 95 % sequence identity. This identity with the contribution of *GYPE* results in hybrid molecules that carry one or more novel antigens of the MNS blood group system (Storry *et al.,* 2000; Palacajornsuk, 2006).

1.3.2 Genetic Mechanisms and Expression of MNS Antigens

The antigens of MNS blood group system are expressed on the RBC membrane on sialoglycoproteins which are closely related, GPA and GPB, or hybrids of GPA and GPB (Huang & Blumenfeld, 1991; Palacajornsuk, 2006).

The chromosomal misalignment at meiosis results in genetic mechanisms or events that are responsible for the generation of the variant phenotypes of MNS blood group system. These can be due to unequal homologous recombination (crossing - over) or gene conversion events between the *GYPA* and *GYPB* (homologous genes) in addition to single nucleotide substitution (Table 1.2) (Blumenfeld & Huang, 1997; Palacajornsuk, 2006).

On one hand, the *GYPA* and *GYPB* express the M or N and S or s antigens, respectively by nucleotide substitution. However, molecular studies of the MNS blood group system revealed that the expression of M and N antigens is dependent on 2 amino acid substitutions encoded on exon 2 of the *GYPA*. Whereas, Ss polymorphism of *GYPB* depend on single amino acid substitution (Table 1.2) (Kudo *et al*, 1990; Huang &

Blumenfeld, 1991; Blumenfeld & Huang, 1997; Nakayashiki *et al*, 2003; Palacajornsuk, 2006).

Table 1.2: Molecular mechanisms of MNS blood group system antigens, Palacajornsuk, (2006).

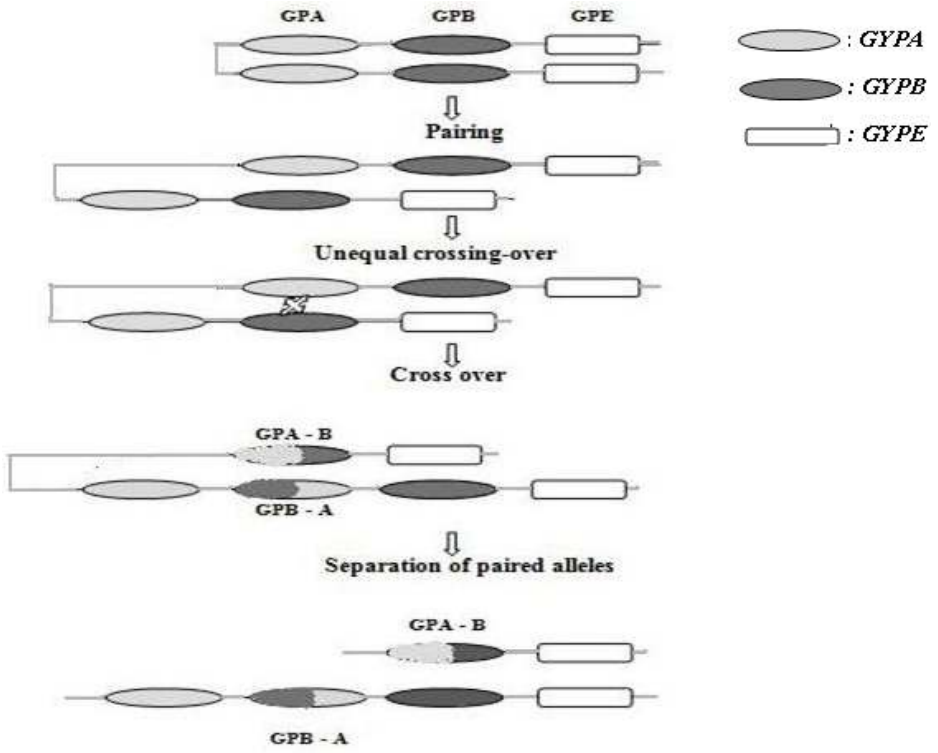
Molecular mechanisms	Associated antigens
Single nucleotide substitution	GPA: Vr, Mt^a, Ri^a, Ny^a, Or, ERIK, Os^a, ENEP/HAG, ENAV/MARS GPB: S/s, M^v, s^D, Mit
Two or more nucleotide substitution	M/N
Unequal crossing over	St^a, Dantu, Hil, TSEN, MINY, SAT
Gene conversion	He, Mi^a, V^w/Hut/ENEH, Mur, M^g, M^c, St^a, Hil, Hop, Nop, DANE, MINY, MUT

On the other hand, both unequal crossing over and gene conversion events give rise to hybrid sialoglycoproteins comprising part of GPA and part of GPB which provide an explanation for the great genetic and antigenic diversity within the MNS blood group system.

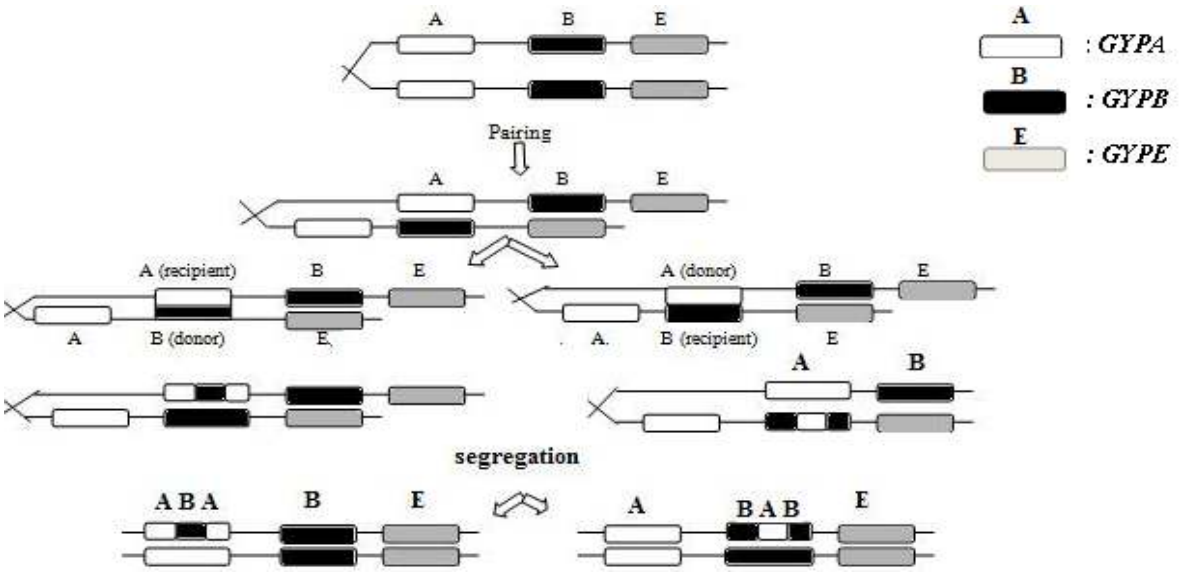
Firstly, the unequal crossing over (Fig.1.2 A) is a mutual exchange of nucleotides between misaligned homologous genes during meiosis. This mechanism occurs between regions of homology with generation of two recombinants in reciprocal arrangement. Crossing-over may occur in two ways; resulting either in gene contraction or expansion. For instance, in the case of the homologous recombination between *GYPA* and *GYPB* (Fig.1.2 A), gene contraction accompanied the formation of *GYP* (*A-B*) hybrid genes upstream of *GYPE* and the gene expansion is characteristic of *GYP* (*B-A*) hybrid genes which is usually accompanied by the three possible genes.

Secondly, for the gene conversion (Fig. 1.2 B) a portion of one gene can replace a homologous part of the other gene and does not result in a reciprocal product. For example, in the case of *GYP* (*B-A-B*) and *GYP* (*A-B-A*) hybrid genes (Fig. 1.2 B), the gene conversion can cause insertion of nucleotides from *GYPA* (as the donor) into the *GYPB* (recipient) and *GYPB* (donor) into the *GYPA* (recipient) respectively. After transfer, the donor gene becomes repaired, and the recipient contains the exchanged part of the donor gene and turns into the hybrid gene.

Lastly, those two mechanisms of recombination events between glycophorin genes, gene conversion and unequal homologous crossing-over produce hybrid genes that encode novel glycophorin molecules carrying certain low-incidence antigens in the MNS blood group system (Blumenfeld & Huang, 1997; Palacajornsuk, 2006).



(A) Unequal homologous recombination (crossing-over) of glycophorin genes



(B) Gene conversion events of glycophorins genes.

Fig. 1.2 Genetic rearrangements of glycophorin genes, resulting in the formation of glycophorin hybrid alleles that encode novel glycophorin molecules carrying certain low-incidence antigens in the MNS blood group system. (Adapted from Blumenfeld & Huang, 1997).

1.3.3 Molecular Basis of Mi subsystem antigens

Molecular studies have shown that the Mi subsystem antigens of the MNS blood group system are expressed on *GYP (A-B)*, *GYP (A-B-A)* and *GYP (B-A-B)* hybrids gene (Table 1.3). However, GP.Hil (Mi.V) and GP.JL (Mi.XI) are encoded by a *GYP (A-B)* hybrid gene. The RBCs carrying GP.Hil express the MINY antigen, while those carrying GP.JL express TSEN and MINY antigens. In addition, the GP.Vw (Mi.I), GP.Hut (Mi.II), GP.Nob (Mi.VII), GP.Joh (Mi.VIII) and GP.Dane (Mi.IX) each are encoded by a *GYP (A-B-A)* hybrid gene. The small inserts range from 1 to 16 bp and do not alter the open reading frame or disrupt the splice sites. Therefore, the short part of the pseudoexon is translated in this hybrid gene (Huang & Blumenfeld, 1991-a; Reid & Lomas- Francis, 2004; Palacajornsuk, 2006). The insert encoding GP.Vw and GP.Hut variants results in an amino acid polymorphism at position 28; the threonine present in GPA is replaced by methionine in the case of GP.Vw and lysine in the case of GP.Hut (Huang *et al*, 1992-b, Palacajornsuk, 2006).

The structural studies of GP.Nob demonstrated that it results from GPA amino acid substitutions at residues 49 and 52, in which the arginin at position 49 in GPA is replaced by threonine and the tyrosine at position 52 is exchanged by serine due to the replacement of ten nucleotides (nt 67-76) in exon 3 of *GYPA* by the corresponding sequence of *GYPB* pseudoexon (Dahr *et al,* 1987; Skov *et al*, 1991; Palacajornsuk, 2006).

Table 1.3: Molecular basis of Miltenberger subsystem antigens: Hybrid glycophorin molecules, phenotypes and associated antigens (Reid & Lomas- Francis, 2004).

Molecular basis	Glycophorin	Phenotype symbol	Associated antigen
GYP (A-B)	GP (A-B)	GP.Hil (Mi.V) GP.JL (Mi.XI)	Hil, MINY TSEN,MINY
GYP (*B-A*)	GP (B-A)	GP.Sch (M^r) GP.Dantu	St^a Dantu
GYP (B-A-B)	GP (B-A-B)	GP.Mur (Mi.III) GP.Bun (Mi.VI) GP.HF (Mi.X) GP.Hop (Mi.IV)	Mi^a, Mur, MUT, Hil, MINY Mi^a, Mur, MUT, Hop, Hil, MINY Mi^a, MUT, Hil, MINY Mi^a, Mur, MUT, Hop,TSEN,MINY
GYP (A-ψB-A)	GP (A- B-A)	GP.Vw (Mi.I) GP.Hut (Mi.II) GP.Nob (Mi.VII) GP.Joh (Mi.VIII) GP.Dane (Mi.IX)	Mi^a, Vw Mi^a, Hut, MUT Nob Nob, Hop Mur, Dane

ψ: Pseudoexon

RBCs with GP.Joh express Joh antigen. Moreover, the GP.Joh (Mi.VIII) is nearly similar to GP.Nob but it differs in: (1) the GP.Joh is associated with the Hop as well as the Nob antigen (2) The altered GPA of GP.Joh differs from GP.Nob by having single amino acid substitution at residue 49 (arginine to theronine). Similarly, the RBCs carrying GP.Dane (Mi.IX) express Mur and DANE antigens. (Skov *et al*., 1991; Huang *et al.,* 1992-a*;* Palacajornsuk, 2006).

For the GP.Mur (Mi.III), GP.Hop (Mi.IV), GP.Bun (Mi.VI) and GP.HF (Mi.X) of Mi subsystem phenotypes are each encoded by a *GYP (B-A-B*) hybrid gene. Firstly, the GP.Mur is nearly similar to GP.Bun but differ in: (1) GP.Bun cells are Hop positive (2) the allele responsible for GP.Bun is aligned with Ms, whereas that for GP.Mur always travels with s antigens either as Ms or Ns. In addition, the *GYP.Bun* gene differs from the *GYP.Mur* gene by only one nucleotide in the coding sequence. Secondly, GP.Hop is identical to GP.Bun but it expresses TSEN and not Hil. Finally, the GP.HF is similar to GP.Mur and GP.Bun. In addition, the *GYP (B-A-B)* hybrid express GP.HF which created by a 98-bp insert from exon 3 of *GYPA*, encodes a peptide differing from GP.Mur and GP.Bun by five and six amino acid residues, respectively (Huang & Blumenfeld, 1991-b; Story *et al*., 2000; Palacajornsuk, 2006).

1.4 Detection of Mi^a Phenotype

In 2001, Chen *et al*. was the first to report the production and characterization of a murine monoclonal antibody (mAb), anti-Mi^a - GAMA210 which can be used to confirm the existence of the Mi^a antigen. In addition, they reported that this mAb (GAMA210) reacts by direct agglutination and specifically agglutinates Mi.I, Mi,II, Mi. III, Mi.IV, Mi.VI and Mi.X RBCs, but not with RBCs of the Mi.V, Mi.Vll, Mi.VIII, Mi.IX and Mi.XI phenotypes (Table 1.4). Palacajornsuk, (2006) mentioned that some classes of Mi did not react with anti-Mi^a but reacted with one or more of the other three specific antisera, e.g., Mi.V RBCs reacted with anti-Mi^a but did not react with anti-Hil (Palacajornsuk, 2006).

Table 1.4: Serologic testing of monoclonal anti-Mi^{a} (GAMA210). (Adapted from Chen *et al.,* 2001).

Mi Antigens		**Reaction of RBCs with antiserum to the following antigens**					**Direct agglutination with GAMA210**	
Mi.	GP.	Mi^{a}	Vw	Hut	Mur	MUT	IS	30 min RT
Mi.I	GP.Vw	+	+	-	-	-	3+	3+
Mi.II	GP.Hut	+	-	+	-	+	3+	3+
Mi.III	GP.Mur	+	-	-	+	+	3+	3+
Mi.IV	GP.Hop	+	-	-	+	+	2+ ^{s}	3+
Mi.V	GP.Hil	-	-	-	-	-	0	0
Mi.VI	GP.Bun	+	-	-	+	+	2+ ^{s}	3+
Mi.VII	GP.Nop	-	-	-	-	-	0	0
Mi.VIII	GP.Joh	-	-	-	-	-	0	0
Mi.IX	GP.Dane	-	-	-	+	-	0	0
Mi.X	GP.HF	+	-	-	-	+	3+	3+
Mi.XI	GP.JL	-	-	-	-	-	0	0

IS: Immediate spine, RT: room temperature, 2+ ^{s}: strong reaction, Mi: Miltenberger, GP:Glycophorin.

1.5 Prevalence of Mi Subsystem in Asia

The MiIII (GP.Mur) phenotype of Mi subsystem is common in Asia (Broadberry & Lin, 1994). The incidence of the MiIII (GP.Mur) phenotype is commonly seen in China and Taiwan in addition to South-east Asia populations especially along the South-east Asia coast lines of China and Taiwan (Prathiba, *et al*., 2002). The GP.Mur phenotype has been found among Chinese populations in different geographic locations in Asia; 7.3 % among Chinese in Taiwan, 6.28 % in Hong Kong Chinese blood donors (Broadberry & Lin, 1994), and also has been found in Thailand with a percentage of 9.7 in Thai blood donors (Chandanayingyong & Pejrachandra, 1975; Broadberry & Lin, 1994). In addition, Blumenfeld & Huang (1997) discussed that the studies on Mi genetic polymorphism have been extended to those human populations that now have become accessible worldwide, for example, the incidence of MiIII in Taiwan varies among different ethnic groups and may reach up to 90% in some regions.

1.6 Clinical Significance of MNS Antibodies

1.6.1 Transfusion reactions

For the MNS antibodies, some cases of hemolytic transfusion reaction (HTR) have been reported due to anti-M and anti-N in addition to anti-S and anti-s (Erskine & Wladyslaw, 1978; Sancho *et al.,* 1998; Guastafierro *et al.,* 2004*)*. Moreover, a transfusion reaction due to anti-U also has been described previously (Rothman *et al.,* 1976).

The Miltenberger subsystem antibodies are known to be clinically significant. Anti-Mi^a has been reported to cause of transfusion reactions, for instance; Lin & Broadberry (1994) reported a case of intravascular transfusion reaction in a Taiwanese patient due to anti-Mi^a; that also has been mentioned by Parthiba *et al*. (2002). Similarly, a case of HTR has been reported in Hong Kong by Cheng *et al*. (1995). Intravascular hemolytic transfusion reactions that due to other antibodies of Mi subsystem also have been mentioned, for example, anti-Vw has been reported in transfusion reaction cases (Molthan, 1981).

1.6.2 Hemolytic Disease of the Newborn (HDN)

Both anti-S and anti-s of the MNS antibodies are capable of causing hemolytic disease of new born (HDN). Less common causes HDN include anti-M, anti-N and anti-U have been reported previously (Dopp & Isham, 1983; Broadberry & Lin, 1994; Duguid *et al*, 1995).

Other antibodies to low-incidence MNS antigens have been implicated in HDN cases, for instance, Rearden *et al*. (1987) reported a severe case of HDN due to anti-Vw. Similarly, Gorlin *et al*. (1996) reported a case of HDN due to anti-Vw. In addition, cases due to anti-Hut and anti-Hil also have been mentioned. Moreover, Broadberry & Lin (1994) reported cases of HDN due to anti-Mi^a and they proposed the importance of including the MiIII phenotype in pre-transfusion antibody screening in Taiwan. Similarly, Lin *et al.* (1996) reported the first case of HDN due to anti-Mur in Hong Kong. In addition, Van & Steiner (2004) documented the first case of severe HDN caused by anti-MUT and a case of hydrops foetalis due to anti-Mi^a was also reported (Wu, 2002).

1.7 Detection of anti - Mi^a In Malaysian Populations

As mentioned previously, the antibodies of the Mi subsystem are clinically significant in Asian populations. Since the Malaysian population is composed of multi-ethnic groups; comprising Malays, Chinese and Indians in addition to other groups, Parthiba *et al.* (2002) analyzed and detected the presence and frequency of GP.Mur (Mi III) phenotype and the corresponding antibodies at University of Malaya Medical Center (UMMC), Kuala Lumpur, Malaysia; using serological techniques. The frequency of antibody detection is 0.2%, 0.3% and 0.2% respectively. Similarly, the prevalence of the GP.Mur red cell phenotype was found in 4.9% of the Chinese, 2.8% in Malays and 3.0% in the Indians. Thus, they recommended that we should include GP.Mur phenotype RBCs in the antibody screening cells and panel cells used in Asian populations.

1.8 Objective of the Study

In this study, the main goal was to screen for, detect and compare the prevalence of the common variants of the Miltenberger subsystem of the MNS blood group system that are prevalent within blood donors in a multi-ethnic population by using polymerase chain reaction-sequence specific primer (PCR-SSP) technique.