Re-evaluation of Modified Rapid Monoclonal Antibody Immobilization of Platelet Antigen (MR-MAIPA) For Detecting Platelet Antibody In Patients With Suspected Immune Thrombocytopenia

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DECLARATION

I hereby declare that this research has been sent to Universiti Sains Malaysia for the degree of Masters of Medicine in Transfusion Medicine. It is also not to be sent to any other universities. With that, this research might be used for consultation and will be photocopied as reference.

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Dr Mohd Fahmi Bin Aziz

PIPM 0052/11

DEDICATION

This dissertation is dedicated to my beloved wife, Junaidah Binti Mat for her support, patience and understanding during this challenging four years; to my lovely children, Muhammad Syakir Irfan and Luqman Hadif, as they have given me courage and happiness; and to my parents and family for their endless love, prayers and encouragement.

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ABBREVIATIONS

AMDI	Advanced Medical and Dental Institute
BSA	Bovine Serum Albumin 30%
BTS	Blood Transfusion Service
ELISA	Enzyme-linked immunoabsorbent assay
GAH	Goat anti-human
GAH-G	Goat-anti-human IgG
GAH-M	Goat anti-human IgM
GAM	Goat anti-mouse
GP	Platelet membrane glycoprotein
HLA	Human Leukocyte Antigen
HPA	Human Platelet Antigen
IMR	Institute of Medical Reseacrh
IVIg	Intravenous Immunoglobulin
Mab	Monoclonal antibody
MAIPA	Monoclonal Antibody Immobilization of Platelet Antigen
MREC	Medical Research and Ethic Committe
MR-MAIPA	Modified Rapid-Monoclonal Antibody Immobilization of Platelet Antigen
NaCl	0.9% Saline

NC	Negative control serum
NBCKL	National Blood Centre Kuala Lumpur
PBS	Phosphate Buffered Saline
PC	Positive Control serum
SOP	Standard Operating Procedure
SPSS	Statistical Package for Social Sciences
TBS	Tris Buffer Solution
USM	Universiti Sains Malaysia
WC	Weak Positive Control serum
WHO	World Health Organization

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ABSTRAK

Kajian Penilaian Semula *Modified Rapid Monoclonal Antibody Immobilization of Platelet Antigen* (MR-MAIPA) Dalam Mengesan Antibodi Terhadap Platelet Di Kalangan Pesakit Pesakit Yang Di Suspek Mengalami *Immune Thrombocytopenia*

Pengenalan. Ujian antibodi terhadap platelet di Malaysia hanya dijalankan di Pusat Darah Negara (PDN), Kuala Lumpur. Sebelum tahun 2013, ujian antibodi terhadap platelet di Malaysia dijalankan menggunakan teknik MAIPA. Namun begitu, MAIPA memerlukan masa selama tiga hari untuk setiap ujian dijalankan. Ini menyebabkan kelewatan dalam mengenal pasti antibodi terhadap platelet sekaligus boleh menyebabkan doktor yang merawat pesakit mengalami sedikit kesukaran dalam merawat pesakit. Oleh itu, sejak Julai 2013, Pusat Darah Negara telah menggunakan MR-MAIPA untuk menjalankan ujian mengenal pasti antibodi terhadap platelet. Semenjak pelaksanaan MR-MAIPA, tiada penilaian semula mengenai teknik ini.

Kajian ini bertujuan menilai semula MAIPA dan MR-MAIPA dalam megesan antibodi tehadap platelet.

Kaedah. Kajian ini dijalankan secara propektif di mana semua sampel yang dihantar ke Pusat Darah Negara untuk identifikasi antibodi platelet, bermula 1 Jun 2015 sehingga 30 November 2015 telah diambil untuk kajian. Proses identifikasi antibodi platelet bagi sampel tersebut dijalankan menggunakan MAIPA dan MR-MAIPA secara serentak. Untuk MR-MAIPA, ujian tambahan dijalankan dengan pentitratan kawalan positif (sehingga pencairan 1:256) untuk meniliai had sensitiviti MR-MAIPA. Kesemua data yang didapati dari kajian ini akan dianalisa untuk sensitiviti, kekhususan, Nilai Ramalan Positif (*Positive Predictive Value*) dan Nilai Ramalan Negatif (*Negatif Predictive Value*).

Keputusan. Terdapat 40 sampel dari 40 pesakit yang berlainan terlibat dalam ujian penilaiaan MR-MAIPA dan MAIPA. Dari 40 sampel tersebut, 77.5 peratus adalah negatif untuk antibodi terhadap platelet dan hanya 22.5 peratus sahaja adalah positif untuk antibodi terhadap platelet, untuk kedua-dua ujian tersebut,. Sensitiviti, spesifisiti, Nilai Ramalan Positif (*Positive Predictive Value*) dan Nilai Ramalan Negatif (*Negatif Predictive Value*) MR-MAIPA adalah setara dengan MAIPA. Untuk sensitiviti MR-MAIPA, ia mampu mengesan Anti HPA-1a dan Anti HLA-1 sehingga pencairan 1:256. Bagi Anti HPA-3a, MR-MAIPA hanya mampu mengesan sehingga pencairan 1:8.

Kesimpulan. MR-MAIPA adalah setara dengan MR-MAIPA dalam mengenal pasti antibodi terhadap platelet. Namun begitu, MR-MAIPA mempunyai kelebihan dari segi tempoh masa ujian iaitu 5 jam berbanding MAIPA yang 8 jam. Oleh itu, MR-MAIPA sesuai digunakan sebagai ujian mengenal pasti antibodi terhadap platelet. Namun begitu, platelet panel yang digunakan oleh kedua-dua teknik harus dipantau dengan kerap untuk memastikan kejituan teknik ini dalam mengesan antibodi terhadap platelet.

Kata kunci: MAIPA, MR-MAIPA, Pusat Darah Negara

ABSTRACT

Re-evaluation of Modified Rapid Monoclonal Antibody Immobilization of Platelet Antigen (MR-MAIPA) For Detecting Platelet Antibody In Patient With Suspected Immune Thrombocytopenia

Introduction. Platelet antibody identification test in Malaysia is solely run by the National Blood Centre, Kuala Lumpur (NBCKL). Before 2013, the antibody identification test was using Monoclonal Antibody Immobilization of Platelet Antigen (MAIPA). However, MAIPA needs three days to complete the test. This may cause delay in detecting platelet reactive antibody, hence some inconveniences for clinician in treating their patients. Therefore, since July 2013, NBCKL had been utilizing MR-MAIPA for identifying platelet reactive antibody. Since MR-MAIPA implementation, no further re-evaluation of the method had been done.

This study aims to evaluate MAIPA and MR-MAIPA in detecting the platelet antibody in NBCKL.

Methods. A prospective study was conducted in which all samples sent to NBCKL from 1st June 2015 - 30th November 2015 for platelet antibody identification was included in the study. The sample is run both using MAIPA and MR-MAIPA concurrently. For MR-MAIPA, further evaluation was carried out, a titration of positive control (up to 1:256 dilution) was titrated to evaluate sensitivity of MR-MAIPA. All the data collected was analyze for specificity, sensitivity, Positive Predictive Value (PPV) and Negative Predictive Value (NPV).

Results. There were 40 samples from 40 different patients. Out of the 40 samples, 77.5% were negative for platelet reactive antibody and only 22.5% were positive for platelet reactive antibody, for both MR-MAIPA and MAIPA. The sensitivity, specificity, PPV and NPV of MR-MAIPA were comparable with MAIPA. For sensitivity, the MR-MAIPA was able to detect Anti-HPA 1a and Anti-HLA 1 up to dilution of 1:256. For Anti-HPA- 3a, MR-MAIPA was able to detect up to 1:8 dilution.

Conclusions. The MR-MAIPA had equivalent sensitivity as MAIPA in detecting platelet reactive antibody. The MR-MAIPA has the advantage of completing the test within 5 hours to MAIPA that needs 8 hours to complete the test. The MR-MAIPA performance is suitable to be used for platelet antibody identification. However, the platelet panel used in both method need to be regularly monitored to ensure the test is precise in detecting platelet antibodies.

Key words: MAIPA, MR-MAIPA, National Blood Centre, Kuala Lumpur (NBCKL)

CHAPTER 1

INTRODUCTION

1.1 Overview of platelet serology service in Malaysia

The Platelet serology Unit, under Immunohematology Department, National Blood Centre Kuala Lumpur (NBCKL) was first established in 1999. The tests offered were platelet antibody screening, platelet antibody identification and platelet cross matching. Investigation to diagnose patient with thrombocytopenia: from Neonatal Alloimmune Thrombocytopenia (NAIT), Idiopathic Thrombocytopenic Purpura (ITP) and Platelet Refractoriness would benefit from these tests. The request for platelet antibody identification had been steadily increased every year. This increment is demonstrated by the accumulative number of sample per year – 89 samples in 2012 to 101 samples in 2014. The factors contributing to the increment are due to increased number of patients, improvement in the current health facilities and increased awareness of the clinician on such conditions and diagnoses.

Few methods of platelet antibody detection were introduce and used: Immucor Capture P®, Solid Phase Red Cell Adherence with Frozen Platelets (SPRCA-F) and Monoclonal Antibody Immobilization of Platelet Antigen (MAIPA). MAIPA has been identified as the gold standard. Despite been identified as the gold standard, it has few limitations that can influence the management of patient.

1.2 Overview of MAIPA and MR-MAIPA

Several techniques had been used worldwide for platelet antibody identification. There were techniques that involving stripping of HLA antigen from platelet using chloroquine pre-treatment, solid phase radio immune assay and Western blot procedure. However, each of these techniques has its disadvantages such as indirect detection of platelet reactive antibody, destroying HLA antigen and insensitiveness in detecting the antibody. In 1987, Kiefel and colleague had made a discovery in platelet antibody identification technique. It was an enzyme immunoassay that utilizes highly platelet-specific monoclonal antibody. It was a superior and sensitive tool for detailed analysis of sera with ambiguous serological findings. The technique was named as Monoclonal Antibody Immobilization of Platelet Antigen, or better known as MAIPA (Kiefel *et al.*, 1987). For the next 20 years, MAIPA became a gold standard for platelet antibody identification.

Although MAIPA is the gold standard in platelet antibody identification, the test was time consuming, causing a major concern. These may cause diagnostic delay, thus in 2007 a group of researchers had made a modification to the MAIPA technique. Campbell *et al*. found that MAIPA took approximately 8 hours to complete a single test and substantial variations in its protocol, which is causing interlaboratory differences in specificity and sensitivity, needs to be addressed. A new technique should be faster and at least equivalent as the original MAIPA in term of sensitivity and specificity in platelet antibody identification needs to be developed. Campbell *et al.* made a modification to the original MAIPA, which resulted in an assay that is much faster without losing its sensitivity. The technique is described as Modified Rapid Monoclonal Antibody-Specific Immobilization of Platelet Antigen, or better known as MR-MAIPA. This method can be done within 5 hours compared to MAIPA which took 8 hours, without loss of sensitivity. With this improvement, diagnosis can be made faster, and patient can receive better treatment (Campbell *et al.*, 2007)

1.3 Rational justification of the study

MR-MAIPA has been evaluated and compared before. Only the evaluation was not done in NBCKL. No correlation had been done in evaluating MR-MAIPA against MAIPA in detecting platelet antibody identification. A good correlation finding is a value in quality assurance of a test.

1.4 Objectives

1.4.1 General Objective

To evaluate the effectiveness of MR-MAIPA and MAIPA in platelet antibody identification.

1.4.2 Specific Objectives

i. To investigate the limit of sensitivity of MR-MAIPA in platelet antibody identification.

ii. To evaluate the performance of MAIPA and MR-MAIPA

iii. To describe the antibodies detected by MAIPA and MR-MAIPA.

1.5 Research Hypothesis

1.5.1 Alternative Hypothesis

MR-MAIPA technique is equivalent or superior to reference method/gold standard MAIPA in platelet antibody identification.

1.5.2 Null Hypothesis

MR-MAIPA is less superior then MAIPA in detecting platelet antibody.

CHAPTER 2

LITERATURE REVIEW

2.1 Platelet overview

Platelet is an important part of haemostasis. Early of its discovery, it was merely known as dust of the blood. Started in the bone marrow, platelets are form from the fragmentation of the mature megakaryocytes (Kaushansky, 2008).

Mature platelet is anucleate, with a discoid shape and a dimension of approximately 3.0 x 0.5um. Since the platelet has no nucleus, the lifespan of the platelet is short with duration of 9 to 10 days (Hanson SR - Slichter, 1985). It has four main storage granules which are the dense granules, α-granules, peroxisomes and lysosomes. The dense granules and the α-granules content are important in supporting haemostasis. The dense granule contains high level of ADP, ATP, Serotonin and Ca2+. The α-granules on the other hand contain the Fibrinogen, Von Willebrand Factor (vWF), Factor V, Plasminogen, PAI-1, α2-antiplasmin and much more (Watson and Harrison, 2010).

In an injury, where there is bleeding, the platelet would first form the platelet plug, which later followed by coagulation process. The traditional thoughts of coagulation would involve the intrinsic and extrinsic pathway of coagulation factors. However, with the cell based theory of coagulation, it was shown that coagulation is not just about cascade of coagulation factors. Platelet also plays an important role in haemostatic activation. It serves as a stage for the coagulation factors to react on the surface. In summary, in the cell based theory, there are three main phases towards coagulation, namely, initiation, amplification and propagation phase. In initiation phase, exposed Tissue Factor (TF) from injury vessel to the flowing blood resulted in generation of small amount of Factor IXa and thrombin. This brings to the second phase, which is amplification phase, where these small amounts of thrombin

will then activates the platelets, which releases vWF and leads to formation of Factor Va, Factor VIIIa and Factor Xia. In the final phase, Propagation phase, those various activated coagulant factors that generated in earlier phase, assemble on the activated platelet to form tenase complex, leading to Factor Xa generation. Later, the prothrombinase complex forms, resulting in burst of thrombin generation (Smith, 2009).

Human platelets express blood group antigen (eg: ABO, I, P), CD 36, HLA-1, GP VI and its own antigen, the Human Platelet Antigen-HPA (Cooling, 2007; Wu, 2014). Until now, there are 34 HPA have been identified which are expressed on 6 different glycoproteins. The antigen expression varies from ethnics and populations (Wu, 2014). The variety of HPA may lead to alloimmunization, especially if the recipient has antigen negative towards the donor antigen. The pathophysiology of platelet alloimmunization is also the same in the case of Foetal and Neonatal Alloimmune Thrombocytopenia (FNAIT), where the mother developed antibody towards the foetal and neonatal platelet (Koh *et al.*, 2012; Bertrand and Kaplan, 2014). Other conditions that have the same principle as above is Post Transfusion Purpura (PTP) and post transfusion platelet refractoriness (Norton *et al.*, 2004; Keashen-Schnell, 2007).

2.2 Immune Thrombocytopenia

In any immune thrombocytopenia, such as Neonatal Alloimmune Thrombocytopenia (NAIT), Post Transfusion Purpura (PTP), platelet transfusion refractoriness or Immune Thrombocytopenic Purpura (ITP), they share a common cause, platelet-reactive antibodies (Metcalfe, 2004; Sandler, 2004; Sathar, 2006; Keashen-Schnell, 2007; Bertrand and Kaplan, 2014).

2.2.1 Neonatal Alloimmune Thrombocytopenia (NAIT)

The pathophysiology of NAIT is when maternal develops antibody toward foetal platelet antigen which the foetal inherited from the paternal side. NAIT may cause severe bleeding for the foetus and also subsequent pregnancies if the foetal poses the platelet antigen which the mother had develop antibody against it (Williamson *et al.*, 1998; Kaplan, 2007). The NAIT can manifest from petechial up to intracranial haemorrhage. NAIT can occur as early 20-24 weeks of gestation (Williamson *et al.*, 1998). The severity of NAIT may differ among anti HPA. Anti HPA-1a and Anti HPA-3a is usually associated with severe NAIT while Anti HPA-5b is less severe than Anti HPA-1a (Kaplan, 2007). The prevalence of NAIT is around 1 in 800-1000 neonates (Bertrand, 2014). Some journal would quote the prevalence is 1 in 1000-2000 live births (Hacene Brouk, 2015). Other study would quote up to 1 in 1000-5000 (Ng Khuen Foong, 2011). In 2011, there was a case report in Malaysia regarding NAIT which demonstrate the maternal platelet reactive antibody against paternal platelet antigen inherited by the foetal. In the paper, it was mentioned that Anti HPA-1a is the cause of the NAIT (Ng Khuen Foong, 2011). In 2015, another paper demonstrate of Anti HPA-1a in Malaysia (M. I. Armawai, 2015). Anti HPA-1a is more common cause of NAIT in Caucasian rather than Asian. Anti HPA-4b is much more common in East Asian (Nadarajan, 2014). Therefore, the finding of Anti HPA-1a as the cause of NAIT in these two cases in Malaysia is a rare finding. Since Anti HPA-1a is more common in Caucasian, some European country (eg: Norway), carried out routine investigation for maternal Anti HPA-1a antibody level as a potential predictor of alloimmune thrombocytopenia in the newborn (Killie *et al.*, 2008)

In the treatment of NAIT, ideally, antigen negative platelet towards the detected platelet alloantibody detected should be given to the neonate. Therefore, the platelet from the mother of the affected neonate may be used for the treatment. If, these platelet are use, it

should be washed to remove the platelet alloantibody and irradiated to prevent Transfusion Associated Graft Versus Host Disease (Bertrand and Kaplan, 2014; Hacene Brouk, 2015). However, this might not be available due to some constraints, therefore, registry of HPA typed donor had been developed in some countries, to find compatible platelet. Some country, such as France, even used frozen platelet for treatment modality. However, in emergency situation, a random platelet can be used with addition of IVIG to limit its destruction (Sachs, 2013; Bertrand and Kaplan, 2014; Hacene Brouk, 2015). In one case reporting, it had shown successful transfusion of incompatible donor platelets in NAIT case. The transfusion of incompatible donor platelet was due to delay in MAIPA reporting for the patient (Schallmoser *et al.*, 2006).

2.2.2 Immune Thrombocytopenic Purpura (ITP)

ITP is an autoimmune disorder in which there is a persistent thrombocytopenia (typically platelet count is less than 150 x109/L). It can affect adults and also children (Norton *et al.*, 2004; Sathar, 2006). The prevalence of the disease ranges from 4-5.3 in 100000 in children. In the US, the incidence ranges from 5.8-6.6 in 100000 populations (Force, 2003; Sathar, 2006). In ITP, there is no specific or gold standard test to diagnose it. It is a diagnosis by exclusion. Identifying specific platelet antibody might not be useful. However, it may have some values to distinguish immune from non-immune thrombocytopenia in difficult cases (eg: combination of bone marrow failure associated with immune-mediated thrombocytopenia). Recently, there are studies regarding prediction of chronic ITP and risk of bleeding. These studies found that ITP patients with antibody detected through MAIPA may have a risk of bleeding and chronic course of the disease compared to those that have no antibody detected (Grimaldi D, 2014). Patients with platelet count more than $30x10^9$ /L may not need any treatment. Treatment is only started if patient is

symptomatic and platelet count below $30x10^9$ /L. The main objective of the treatment is to reduce the destruction of platelets. The strategies of treatment are divided to First Line and Second Line of treatment. First line treatment is usually involving administering Corticosteroid and IVIG. Second line of treatment involves surgical intervention, splenectomy and medications (eg: Danazol, Azathioprine, Dapsone and Anti –D). For Refractory ITP, multi drugs combination (eg: Vinca alkaloid, Rituximab, IVIG) and eradication of Helicobacter Pylori infection are made to achieve platelet count more than $30x10^9$ /L. Platelet is transfused if there is severe haemorrhage or reached critical value (< 10 x 10^9L) (Sandler, 2001; Force, 2003; Sathar, 2006). Recently, there are studies involving Thrombopoietin (TPO) Receptor Agonist in treating ITP. The outcomes looks promising as patient on the treatment had sustained increased in platelet counts (Gernsheimer, 2009; Provan and Newland, 2015; Rodeghiero and Ruggeri, 2015)

2.2.3 Post Transfusion Platelet Refractoriness

The Trial to Reduce Alloimmunization to Platelets (TRAP) study defined post transfusion refractoriness as increment of less than 5×10^9/L of Corrected Count Increment (CCI) within 1 hour post platelet transfusion on two sequential occasions. This condition may be due to immune or non-immune causes. For immune causes, it is usually due to HLA alloimmunization. However, HPA alloimmunization can also contribute to the cause (Norton *et al.*, 2004; Simon J. Stanworth, 2015). This condition is usually seen in patients who received regular platelet transfusion.

In managing Post Transfusion Platelet Refractoriness case, the cause of refractoriness should be investigated and managed accordingly to the underlying cause. If the cause is due to alloimmunization, an antigen negative platelet should be given to the patient. Other than

that, leukoreduction of blood components may decrease the incident of platelet refractoriness and HLA alloimmunization (Slichter, 1997; Norton *et al.*, 2004; Basire and Picard, 2014)

2.2.4 Post Transfusion Purpura

Post transfusion purpura is defined as severe thrombocytopenia (platelet count < 10×10^9/L) that occurs 5 to 12 days post blood transfusion. A classic presentation would usually involve older, multiparous woman with antibody-mediated drop in platelet after 7 to 10 days after transfusion (Keashen-Schnell, 2007; Mikhail Menis, 2015). It is a rare condition. The Serious Hazard of Transfusion (SHOT) study in the United Kingdom reported the incidence of 1 in 465000 transfusions. Post Transfusion Purpura is usually associated with anti HPA. Treatment of choice would involve giving IVIG with platelet transfusion with a good rapid response may be expected in 85% of cases (Norton *et al.*, 2004).

2.3 Human Platelet Antigen (HPA) and related platelet antibody

Apart from ABO antigen, the platelet also has its own antigen, known as Human Platelet Antigen (HPA). Until today, there are 34 specific platelet alloantigens (HPA 1 to 28bw) had been found (Li *et al.*, 2014). The variety of HPA and its differences from one population to another population pose a possibility of alloimmunization (Kaplan, 2007; Nadarajan, 2014). One of the earliest HPA identified was HPA 1a/1b. It was found in 1956-1957, when a patient develop thrombocytopenia post transfusion (later, it known as post transfusion purpura) (Aster and Newman, 2007). In the Western population, anti HPA 1a is the common cause of NAIT. However, it is rare in the East Asian population. Instead, anti HPA 4b is more common in NAIT cases in East Asian population (Nadarajan, 2014). Although anti HPA 4b is commonly associated with NAIT in the East Asian population, there is still possibility of other rare anti HPA that may cause NAIT. Two of such case had been

reported in Japan, NAIT cases involving anti HPA 21bw (Koh *et al.*, 2012). However, recent study had shown that alloantibodies against low frequency HPA do not account for a significant proportion of NAIT (Santoso and Tsuno, 2015).

As variety of HPA among location and population, a platelet donor registry should be established in each country. This is to provide match platelet to the patients when the need arise (Feng *et al.*, 2006; Tan *et al.*, 2012).

2.4 Platelet Donor Registry

Platelet alloimmunization may cause much problem (Norton *et al.*, 2004) for the patient and the treating physician. The best platelet to be given to a patient who had alloantibody towards platelet is to provide antigen negative platelet (Norton *et al.*, 2004; Bertrand and Kaplan, 2014; Katus *et al.*, 2014). In doing that, two most important components that must work in tandem are the platelet registry and platelet antibody identification. It would be a long arduous task if only one of the components exists and the patient needs the specific platelet transfusion. The data of platelet antigen varies from each region or even country. In almost homogenous population (eg: Japan), it may have different HPA compared to a multi ethnic country (eg: Malaysia). Therefore, each country must develop their own data of platelet registry (Feng *et al.*, 2006; Tan *et al.*, 2012; Li *et al.*, 2014; Nadarajan, 2014; Wu, 2014). Platelet antibody detection should also be utilised efficiently in treating immune causes thrombocytopenia. The development MR-MAIPA had gave a better and faster outcome in detecting platelet antibody (Kiefel, 1992; Campbell *et al.*, 2007; Kaplan *et al.*, 2007). Utilising both platelet donor registry and platelet antibody detection would lead a better outcome in treating immune mediated thrombocytopenia (Nance *et al.*, 2004).

2.5 Platelet Antibody Identification

Platelet antibody identification is important in management of immune thrombocytopenia. In 1987, a study was published on a new technique in platelet antibody identification. It was known as MAIPA. MAIPA had overcome other technical difficulties in platelet antibody identification. The technique based on capturing the trimetric complex (Monoclonal antibody specific to platelet Glycoprotein-Platelet Antigen-Investigated platelet antibody). It is a simple test without needing any sophisticated equipment. However, the test required 8 hours to completed (Kiefel *et al.*, 1987). As the cornerstone of the test is capturing the trimetric complex, the original MAIPA protocol had undergone some modification across the globe. The modifications include the monoclonal antibody used and even the procedure protocol. These variations may cause significant differences in ability to detect antibody level and even the sensitivity of the test itself (Killie MK, 2010). In 2007, a modification to MAIPA which makes it rapid without losing its sensitivity in detecting platelet antibody was discovered. The modifications involved using of smaller sample requirement (investigated serum), reagents and incubation period. It provides a better alternative to the original MAIPA as it can be done much faster, within 5 hours (Campbell *et al.*, 2007)

Although MAIPA had been a gold standard in platelet antibody identification, it has some limitations. For example, the selection of monoclonal antibody for MAIPA may cause competition in binding for the same antigen site as the investigated antibody, resulting in inability to identify the investigated antibody (Tomoya Hayashi and Hirayama, 2015). The preparation of platelet panel, may cause destruction of certain HPA, which may lead to false negative results. In terms of procedure, certain low avidity antibody may be dissociated during wash procedure, also contributing false negative results (Santoso and Tsuno, 2015). Therefore, it is important for a diagnostic lab not to rely on a single technique in their facility to facilitate in investigation of platelet antibody. Recently, a study using flow cytometry

method in detecting platelet antibody had shown 93% concordance with MAIPA finding. It can be done much faster, within 3 hours, with a very small sample of investigated serum needed (10μL of the investigated serum) compared to MAIPA (Leendert Porcelijn, 2013; Santoso and Tsuno, 2015). Another paper had also shown this flow cytometry based method is also recommended for detection autoantibodies in ITP patients (Yunxiao Zhao *et al.*, 2015).

CHAPTER 3

Methodology

3.1 Study Venue

The National Blood Centre Kuala Lumpur serves as the only platelet reactive antibody identification and platelet cross matching centre in Malaysia. It receives request for platelet reactive antibody identification and platelet cross matching from the government hospitals as well as the private health centres.

3.2 Study Duration

The study duration is from 1st June 2015 – 30th November 2015.

3.3 Inclusion Criteria and Exclusion Criteria

3.3.1 Inclusion Criteria

i. All samples for platelet antibody identification received within the study period (1st June 2015 - 30th November 2015).

3.3.2 Exclusion Criteria

i. All rejected samples by NBCKL (eg: lyse sample, insufficient sample, samples in wrong bottle)

3.4 Sample Size

As for a low prevalence disease, sample size for a desired power is chosen in comparing sensitivity of the two tests (MAIPA and MR-MAIPA). The calculation is estimated using G*Power software based on paired design of two-sided McNemar's test. Assuming the proportion of discordant pairs of 0.3 and taking the level of significance (alpha value) of 0.05 and power of 80%, a total of 44 (40 + 10% drop-out rate) positive samples with the disease (by the gold standard) are required to compare sensitivity between the two tests.

Below is the output for the sample size calculation using G*Power:

Exact - Proportions: Inequality, two independent groups (McNemar)

Options: α balancing: α/2 on each side, approximation

Analysis: Sensitivity: Compute required effect size

Input:

Tail(s)	=	Two
α err prob	=	0.05
Power (1-β err prob)	=	0.8
Total sample size	=	40
Prop discordant pairs	=	0.3

The entire sample sent for platelet antibody identification from 1st June 2015 - 30th November 2015 was taken for this study.

3.5 Statistical analysis

Data analysis was done by using the Statistical Package for the Social Science (IBM® SPSS®) version 19. The results of MAIPA and MR-MAIPA will be analyse for sensitivity, specificity, positive predictive value and negative predictive value. The descriptive result of categorical data presented as frequency/percentage.

3.6 Variables definition

3.6.1 MAIPA

Monoclonal Antibody Immobilization of Platelet Antigen also known as MAIPA, is a method in detecting platelet reactive antibody that was discovered by Kiefel in 1987 (Kiefel *et al.*, 1987). In this study, MAIPA is considered as the gold standard of the test.

3.6.2 MR-MAIPA

Modified Rapid Monoclonal Antibody Immobilization of Platelet Antigen also known as MR-MAIPA, is a modification to the original MAIPA in detecting platelet reactive antibody that was discovered by Campbell (Campbell *et al.*, 2007).

3.7 Research tool

A proforma (MAIPA and MR-MAIPA results) was used to document the antibody detected by each method that was used on same sample. A data extraction tool was developed according to the research needs.

3.8 Ethical Issues

Ethical approvals were obtained from the Ministry of Health of Malaysia as current study involved human blood. Current study was registered with the National Medical Research Register (NMRR) with research ID number 22920.

3.9 Test procedures

3.9.1 MAIPA

The MAIPA procedure in this study was based on the working instruction for MAIPA in NBCKL (Ibrahim, 2001)

Day 1

MAIPA procedure is done within 3 days. The summary of the procedure are as follows.

Coating Plate

Microplates were coated with Goat Anti-Mouse antibody (GAM). The coated microplate was seal with parafilm and kept in laboratory fridge. The prepared microplates can be used up to one week post preparation.

Designing worksheet

The worksheet is very important in the procedure. It is where the results are recorded. Basic design of the worksheet can be viewed in the appendix section. All samples must be tested in duplicate and in parallel with a negative control.

Preparation of Platelet Panel Suspension

Platelet panel was prepared as per working sheet. The panel was from donors whom platelet antigens had been genotyped. This will facilitate in the process of identifying the specific platelet reactive antibody later in the test.

Total time used for day 1: The whole day (8 working hours)

Day 2

Sensitization of platelet

Sensitization of platelet depends to the working sheet design. This due to the fact that the platelet prepared for the test was from donor with already known antigen.

A 500µL of the platelet suspension was dispense into 1.5 ml microtubes which already number according to the designed worksheet. These microtubes were then centrifuge for 3 minutes, resulting formation of platelet pellet at the bottom of the microtubes. The supernatant was aspirated and discarded, leaving behind the platelet pellet. 50µL PBS/2% BSA solution was then added to the platelet pellet in the microtubes. 120µl of patient's serum (investigated serum) was later added and incubated at 37^{o}C for 30 minutes. Incubation period may be prolonged to maximum of one hour. After incubation, the mixture was washed using 750µL cold NaCl/0.2%/BSA and 50µL. After washing, Monoclonal Antibody was added (according to the designed worksheet). The platelet was then incubated again at 37^{o}C for 30 minutes. Post incubation, the platelet was washed twice with 750µl cold NaCl/0.2%/BSA solution and centrifuge for 3 minutes. The supernatant was aspirated and discarded, leaving behind sensitized platelet pellet which already forming trimetric complex (Platelet reactive antibody-platelet antigen-Murine monoclonal antibody).

Solubilisation of platelet

The sensitized platelet pellet was ready for solubilisation in order to free the trimeric complex to be captured by GAM later in the process. A 50µl of cold solubilisation buffer was added to the sensitized platelet pellet and incubated at 4ºC for 30 minutes. After incubation, the microtubes were centrifuged for 30 minutes at 4ºC. During the incubation period earlier, blocking GAM plate was done.

Blocking GAM plate

The GAM coated plate that was prepared in Day 1 was taken out of the fridge and washed for 5 times with cold ELISA wash buffer. After washing, 300µL MAIPA wash buffer/0.2% BSA was added to each well. The GAM plate was then incubated at 4ºC for 60 minutes. During the incubation, new 0.8mL microtubes were labelled and filled with 150µL MAIPA wash buffer/0.2%BSA . These microtubes later were use in the following step.

Capture of Antibody Coated GP

The GAM coated microplate that was incubated earlier was removed from the fridge, drained and dried thoroughly.

The solubilised platelet in earlier step was retrieved post centrifugation. The supernatant (platelet lysate) was aspirated and transferred approximately 50µL into newly labelled 0.8mL microtubes that had been prepared earlier. It was then mixed well. After mixing, approximately 50µL of the diluted lysate was transferred into the GAM microplate. 50µL of cold MAIPA wash buffer 0.2% BSA was then added into the microplate. The microplate was then incubated overnight at 4ºC.

If there was urgency, the microplate incubation period may be shorten to 2 hours then followed with ELISA assay which is usually done in Day 3 of the test.

Total time taken for Day 2 procedure is approximately the whole day, including the overnight incubation period. However, it maybe shorten to approximately 6 to 7 hours if the incubation step is shorten to 2 hours only.

Day 3

ELISA Assay

This procedure was carried out after overnight incubation of the GAM plate earlier in Day 2 or after a period of 2 hours incubation in urgent cases.

The Goat Anti Human IgG/IgM (GAH-G/GAH-M) was prepared. The microplate that was incubated earlier was retrieved drained, followed by five times washing with ELISA wash buffer. After washing, the GAH was added to the microplate. The plate incubated in the laboratory fridge for 2 hours. Post incubation, substrate was added to the plate, which will change in colour if the trimetric complex was present. The result is read at 405nm light absorbance, at 15 minutes interval, up to 60 minutes. The final result (at 60 minutes) was then recorded on the MAIPA working sheet.

The total time taken for Day 3 is approximately 4 hours.

As seen in the description of the test above, the MAIPA can be shorten to 2 days or even a day. These depends on the preparation of the GAM plate (as described in Day 1) and incubation of the captured of antibody coated GP (as described in Day 2). The test can be shorten to two working days provided the GAM plate was prepared in Day One and to proceed with the rest of the procedure in Day Two, in which the incubation of the captured of

antibody coated GP is shorten to 2 hours. The MAIPA procedure can be further shorten to a day provided that the GAM plate had been prepared much more earlier prior to the procedure day as the coated GAM plate can be kept up to a week after its preparation.

3.9.2 MR-MAIPA Procedure

The Modified Rapid MAIPA (MR-MAIPA) was first used in NBCKL since 2013. By using the MR-MAIPA, the test duration is shorten to 1 day, compared to previously that was 3 days. The NBCKL MR-MAIPA method is based on the work of Campbell and colleagues, A Modified fast MAIPA for the detection of HPA platelet antigen antibodies: a multi-centre evaluation of a rapid monoclonal antibody specific immobilisation of platelet antigen (MAIPA) assay, which was published in the Vox Sanguinis 2007 (Campbell *et al.*, 2007). The MR-MAIPA used the same principle as MAIPA. The outlines of the MR-MAIPA are as follows:

1. <u>Preparation of Coated Flat-Well (F-Well) Microplate</u>

A 100µL of GAM was added to each well according to designed worksheet. The coated F-well plate is incubated at 4ºC for at least 3 hours. The prepared microplate can be stored up to 2 weeks at 4ºC.

<u>2. Incubation of Platelets with serum</u>

A 100µL of donor platelets was used per well of U well microtitre plate. The U-well microtitre plate was then centrifuge for 3 minutes. The supernatant was discarded, leaving behind the platelet pellet. The U-well microplate was dried on paper towel. After the microtitre plate had dried, 50µL of TBS/BSA was added to each well, resulting in