PLATELET CROSSMATCH BY FLOW CYTOMETRY : EVALUATION AND FEASIBILITY STUDY

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PLATELET CROSSMATCH BY FLOW CYTOMETRY : EVALUATION AND FEASIBILITY STUDY

By

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DECLARATION

I hereby declare that I am the sole author of this thesis entitled "Platelet Crossmatch by Flow Cytometry : Evaluation and Feasibility Study". I declare that this thesis is being submitted to Universiti Sains Malaysia (USM) for the purpose of the award of Master of Science in Transfusion Science. This dissertation is the result of my own research under the supervision of Dr Abdul Rahim Bin Hussein except as cited in the references. The dissertation has been accepted for the study performed and is not concurrently submitted in candidature of any other degree.

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ABBREVIATIONS

ABH	Blood group system
ABO	Phenotype blood group of A, B, AB, or O
AHG	Anti human globulin
CCI	Count corrective increment
CD	Cluster of differentiation
DNA	Deoxyribonucleic acid
EDTA	Ethlenediaminetetraacetic acid
FACS	Fluorescence be activated cell sorting
Fc	Fragment crystallizable
FITC	Fluorescein isothiocyanate
FN	False negative
FNHTR	Febrile non haemolytic transfusion reaction
FNR	False negative rate
FPR	False positive rate
FP	False positive
GP	Glycoprotein
GVHD	Graft Versus Host Disease
HDN	Hemolytic disease of newborn
HLA	Human leukocyte antigen
HPA	Human platelet antigen
ID	Identity
IgG	Immunoglobulin G
IVIG	Intraveneous immunoglobulin

MAIPA	Monoclonal antibody immobilisation od platelet antigen
MOH	Ministry of Health
NAIT	Neonatal alloimmune thrombocytopenia
NMRR	National Medical Research Register
NPV	Negative predicted value
OD	Optical density
PBS	Phosphate buffered saline
PCR-SSP	Polymerase chain reaction – sequence specific primers
PDN	Pusat Darah Negara
PECAM	Platelet endothelial cell adhesion molecule
PIFT	Platelet immunofluorescence test
PPR	Percent platelet recovery
PPV	Positive predicted value
PTP	Platelet transfusion purpura
PTR	Platelet transfusion refractoriness
RES	Reticuloendothelial system
SPRCA	Solid phase red cell adherence
TBE	Tris-borate-EDTA
TN	True negative
TP	True positive
UV	Ultraviolet
°C	Degree Celcius
ul	Microliter
ml	Millilitre
rpm	Radius per minute

ABSTRAK

Pengurusan pesakit yang membentuk kerintangan imun terhadap platelet adalah mencabar. Salah satu kaedah yang ada adalah menerusi pembekalan platelet yang serasi menerusi ujian keserasian platelet. Prosedur ujian yang digunakan iaitu "Solid Phase Red Cell Adherence (SPRCA)" berfungsi dengan baik sebagai ujian keserasian platelet. Walaubagaimanapun, terdapat beberapa kekurangan yang menyebabkan ia kurang sesuai digunakan dalam beberapa keadaan. SPRCA adalah ujian secara manual dan melibatkan intepretasi keputusan ujian dan dokumentasi secara manual sepenuhnya. Disebabkan penggunaan "microplate" berbentuk U, ujian keserasian ini perlu dijalankan secara berkelompok dan tidak sesuai digunakan untuk bilangan ujian yang sedikit. Tambahan pula, operasi manual dan kaedah ujian yang rumit pula boleh menyumbang kepada proses yang berat sebelah. Oleh itu, kecekapan penguji menjadi faktor yang penting. Disebabkan kelemahan-kelemahan ini, terdapat keperluan untuk mencari kaedah ujian lain yang boleh mengatasi kelemahan SPRCA dan seterusnya diimplimentasi ke dalam perkhidmatan tabung darah. "Platelet Immunofluorescence Test (PIFT)" adalah ujian yang menggunakan prinsip ujian yang sama seperti SPRCA tetapi menggunakan flow citometer untuk menganalisis keputusan ujian. Di dalam kajian ini, PIFT diuji untuk mencari kekuatan dan kelebihannya sebagai ujian keserasian platelet, terutamanya pada aspek kualiti prestasi dan ketersediaan untuk operasi makmal. Sebanyak 20 penderma aferesis telah didaftarkan ke dalam kajian ini, dan profil genotip HPA mereka di uji menggunakan ujian PCR-SSP. 11 serum kawalan daripada 4 kategori "platelet reactive antibody" telah dimasukkan ke dalam kajian dan jenis antibodinya dipastikan. Ujian keserasian dijalankan menggunakan suspensi platelet penderma yang baru disediakan, terhadap serum. Setiap ujian keserasian di ulang semula, sama ada pada hari pertama, kedua atau ketiga selepas hari penyediaan suspensi platelet. Ramalan keputusan ujian keserasian di buat berdasarkan maklumat genotip penderma dan jenis antibodi pada serum. Keputusan ujian ramalan ini kemudiannya dibandingkan dengan keputusan sebenar

ujian keserasian yang dilakukan. Keputusan ujian kemudiannya diterjemahkan kepada 'betul' atau 'palsu' dan 'serasi' atau 'tidak serasi', seterusnya dianalisis dengan statistik yang sesuai untuk jenis keputusan dwi-data. Daripada 20 penderma dan 11 serum kawalan, sebanyak 237 ujian keserasian telah dijalankan. Dalam mengenalpasti ketidakserasian, PIFT telah menujukkan keupayaan untuk mengesan sebanyak 91.2%, dengan ketepatan sebanyak 62.4% dan ketidakserasian palsu sebanyak 34.2%. Sebaliknya, semasa mengesan keserasian, pengesanan hanyalah sebanyak 65.8% dengan ketepatan sebanyak 93.2% dengan keserasian palsu hanyalah sebanyak 8.8%. Ini menunjukkan prestasi yang menakjubkan sebagai ujian keserasian platelet dimana ia mengesan lebih banyak ketidakserasian palsu untuk meningkatkan keselamatan dan pada masa yang sama memberikan keputusan serasi yang tepat. Tambahan pula, PIFT boleh dijalankan ke atas bilangan sampel yang sedikit, dan boleh menghasilkan keputusan ujian janaan komputer, dimana kedua-dua ciri ini tidak didapati pada SPRCA. Kos ujian PIFT hanyalah sekitar RM3.50 setiap satu. Ujian ini adalah mudah, murah dan boleh dipercayai. Berdasarkan kajian ini, PIFT telah menunjukkan prestasi yang mengagumkan sebagai ujian keserasian platelet. Ia menawarkan kelebihan yang istimewa dan boleh diserap ke dalam perkhidmatan tabung darah untuk proses pembekalan platelet serasi kepada pesakit yang membentuk kerintangan imun terhadap platelet.

ABSTRACT

Managing patient with immune mediated platelet refractoriness can be very challenging. One of the approach is by selecting compatible platelet via crossmatch test. The existing protocol ; Solid Phase Red Cell Adherence (SPRCA) assay works very well as a platelet crossmatch assay. However, it have some limitations that make it unsuitable to be used in certain situation. SPRCA is a fully manual test with manual result interpretation and documentation. It utilizes U-shape microplate and run on batches of samples. Due to manual operation and laborious procedure, there are chances of bias to occur, therefore competency and skill of operator becomes an important confounding factor. Furthermore, SPRCA is not suitable for single or low throughput crossmatch. Due to these restrictions, there is a need to search for another platelet crossmatch protocol that can overcome the limitations of SPRCA thus be implemented in blood centre setting. Platelet Immunofluorescence Test (PIFT) assay is an assay that runs on similar principle (whole platelet based assay) as SPRCA, except that it utilizes flow cytometer for result analysis. In this study, PIFT is being challenged to discover its strength and advantages in performing platelet crossmatch test, particularly on qualitative performance and feasibility aspect on operational service. 20 platelet apheresis donors were enrolled in this study and their HPA genotype were determine by PCR-SSP. 11 known control serums from 4 category of platelet reactive antibodies were recruited and antibody specificity were determined. Crossmatch were performed on freshly prepared donor's platelet suspension against serum. Each crossmatch were then repeated once either on day-1, day-2 or day-3 of platelet suspension preparation. The predicted compatibility of donor crossmatched to serum were established based on the donor's genotype and antibody specificity, and were compared to the actual crossmatch result obtained. Compatible and incompatible observation were then translated as true and false of positive and negative outcomes. The results were then statistically analysed with parameters suited for qualitative binomial data. From all 20 donors and 11 serums, 237 total crossmatching

have been performed. In detecting incompatibility, PIFT has demonstrated the ability to detect as high as 91.2%, with accuracy of 62.4% and false positive of 34.2%. Conversely, when detecting compatibility, it detects only 65.8% with the accuracy as high as 93.2% with low false negative of 8.8%. This shows an outstanding performance of crossmatching test where it detect more false positive to enhance safety, in the same time gives accurate compatible result. Furthermore, PIFT can be done with single or low throughput crossmatch, and able to generate computer-based-results which a feature that is lacking in SPRCA. PIFT cost about RM3.50 per test. It is a simple, cheap and reliable assay. PIFT shows an outstanding performance as a platelet crossmatch assay. It offers great advantages and are feasible to be implemented in a blood banking setting for the provision of compatible platelet supply for the immune mediated thrombocytopenic patient.

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Chapter 1

INTRODUCTION

1.1 Literature Review

1.1.1 The platelet cell

Platelets, are the smallest blood cells that can be found in the blood circulation. At resting phase, platelet shaped like a discoid lens and can reach the size of 3-5μm at their greatest diameter (Craig H. Fletcher, 2015). However, the size can significantly increase with the formation of pseudopods when they get activated. Despite of their small size that makes them appear as insignificant, they are very crucial to life and are extensively studied for their complex physiology (Rodak et al., 2007).

Platelet are also known as thrombocyte. They are anucleated cells and have short life span of around 10 days. Since it is short, platelet need to be constantly generated from the megakaryocytes in order to maintain a normal platelet level (Cho, 2015). The cell production is regulated by the thrombopoietin hormone and other cytokines. When a megakaryocyte receives response from these thrombopoiesis signals, they release platelet into the blood circulation, with average about 2,000 to 10,000 of platelets per single megakaryocytes (Kaushansky, 2008).

In normal healthy individual, platelet circulates the blood stream in a great amount ranging from 150 to 400 x 10^9/L cell count. In situation when there is an overwhelming of platelet, the haemostatic balance is disturbed and this may lead to blood clotting disorder. This condition is called thrombosis. Conversely, if the platelet count is low, it can trigger spontaneous abnormal bleeding. The low platelet count condition is known as thrombocytopenia. Both conditions can be life threatening and may cause significant morbidity and even mortality to the patient, however thrombocytopenia are more common. It may be resulted from suppression or failure of the bone

marrow to produce the platelet cells, or more commonly through shortened platelet survival due to increase in vivo platelet destruction (Delicou and Bellia, 2015, Lochowicz and Curtis, 2011).

Platelet is well known for its critical involvement in haemostasis with the ultimate goal to crosslink fibrinogen to form fibrin clot thus stopping the bleeding. Later discovery have reveal that they also have role in innate immunity, inflammatory and tumour metastasis (Sorrentino et al., 2015). In active bleeding situation where the autologous platelet is low, transfusion of platelet is necessary to reach the haemostatic control of bleeding. Apart from that, in thrombocytopenic patient, platelet is often transfused as prophylaxis treatment before starting any invasive treatment procedure to the patient (McCullough, 2010). In similarity to the red and white blood cells, platelet also express many antigens on the platelet surface, that could give rise to immunology issues which could hinder the therapeutic effects of a platelet transfusion.

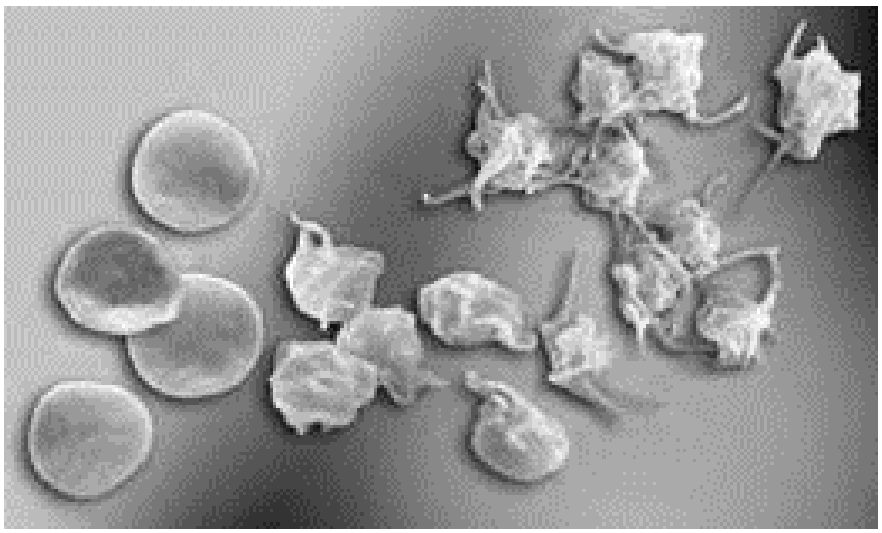


Figure 1 : Platelet morphology.
Picture adapted from The University of Georgia, College of Veterinary Medicine website.
Platelet on the left is at resting stage and platelets on the right is at activated stage.

1.1.2 Platelet alloantigen and alloimmunization

There are two different categories of clinically relevant platelet alloantigens. Antigens that is being shared with other blood cells and tissues are referred to as "Type 1 antigen". The antigens belongs to this type of group is the ABH antigen system that is predominantly found on red blood cells. Another group of antigen are the glycoprotein antigens of the HLA Class 1 which is abundantly found on all nucleated cells. The second category of the platelet alloantigen is the "Type 2 antigen" that are specifically found on platelets (Santoso, 2003, Mueller-Eckhardt et al., 1990), thus are called the platelet specific antigen or the Human Platelet Antigen (HPA).

Antigen are characterised by the capability to elicit an antibody production. When the immune cells of an individual was exposed to a foreign alloantigen, the interaction will provoke sensitization, and later produce the specific alloantibody toward the specific antigen. Upon second exposure to the same alloantigen, the antibody will recognize the specific antigen, form linkage binding and coat the platelet surface. The antibody-coated platelet will soon lead to immune mediated platelet destruction where they are either being destroyed through the complement activation, or through the phagocytosis activity in the reticuloendothelial system (RES). In the blood circulation, as the antibody-coated platelets pass through the spleen and liver of the RES, the fragment crystallisable (Fc) portion of the antibody molecules will engaged to the Fc-receptors on the surface of monocytes or macrophages in these organs. This will finally lead to platelet destruction and clearance (Lochowicz and Curtis, 2011).

Without the capability to induce the formation of alloantibody, a genetically determine protein or carbohydrate will not be called as an "antigen". This will be further explain in paragraph 1.1.2.3 the human platelet antigen group.

1.1.2.1 The ABH blood group on platelet

In blood transfusion service, the inventory management need to be highly efficient in order to keep good coordination of blood stock and blood supply. The pressure mount up with the need to keep low expiry rate of blood, especially for the precious and expensive apheresis unit that only have the shelf life of 5 days. In many blood centres, platelet is transfused without regard to the ABO compatibility, by giving priority to the age of the platelet unit (Kelsey, 2003, Bethasda, 2005). It is widely thought that the amount of the A and B antigen expressed on platelet is too little to significantly affect the survival of the ABO-incompatible platelet in vivo, therefore it is a common practice to transfuse the ABO incompatible platelet (Curtis et al., 2000).

Platelet express the A, B and H antigens on sugar moieties attached to all platelet glycoprotein (Cooling, 2007), however, the majority of ABH is expressed on GPIIb and the platelet endothelial cell adhesion molecule (CD31 and PECAM-1) (Curtis and McFarland, 2014, Curtis et al., 2000). Apart from the ABH antigens, P, Lewis ^{a} and I antigen also appear to be expressed on platelet membrane (Dunstan and Simpson, 1985, Marwaha and Sharma, 2009). However, Rh antigens, which reside on glycoprotein involved in ammonia transport across cell membrane, do not appear to be expressed on human platelets. Neither were the Duffy, Kidd, Kell, and Lutheran antigens (Dunstan et al., 1984). While A1 group is well expressed on platelet that range from 2,100 to 16,000 copies per cell, A2 group express small or no A antigen on platelet (Cooling et al., 2005, Curtis et al., 2000). B expression on platelet was discovered to be weaker than A1 (Cooling et al., 2005, Heal et al., 1989). Since A2 donors have little or no A antigen on platelet membrane, they are compatible with major ABO-mismatch platelet and therefore can be noted as universal platelet donor. This approach have been adopted in Norway where A2 platelet is pooled under the universal group of platelet (Novotny, 2005). About four years later, Julmy and colleagues had done an extensive study on 400 patients of ABO identical, major and minor mismatch platelet transfusion. The result demonstrated that for major mismatch of group A1, the platelet respond

was lower as compared to the ABO identical platelet transfusion. Conversely, major mismatch A2 were as successful as ABO identical transfusion (Julmy et al., 2009) which is in agreement with the previous findings on A2 expression.

Ogaswara and coworkers have discovered that the amount of ABH expressed on platelet membranes differs between individual where each person can be classified as low-expresser and high-expresser group. These 'high expresser group' may have an elevated expression of ~20 times of normal A or B antigen level (Ogasawara et al., 1993). ABO antibodies reacting with the high expresser platelets have been reported to be implicated in NAIT (Curtis et al., 2008).

ABO incompatibility between donor and recipient can be define as major, minor and bidirectional mismatch. This much depending on the A and B antigen expression of the platelet, and also the natural occurring antibody of the suspended plasma (Yazer et al., 2012). In the early years, scientist have reported that, satisfactory increment were obtained with ABO compatible platelet (Ogasawara et al., 1993, Skogen et al., 1988, Brand et al., 1986). However, more aggressive studies have been done (Stolla et al., 2015) and many reports from different group of researchers have been published regarding the observation on poor platelet transfusion increment associated to the transfusion of ABO-incompatible blood (Refaai et al., 2011, Inaba et al., 2010, Shanwell et al., 2009). It is soon to be understood that the A and B antigen on the platelet does have an impact on the survival of the transfused platelet as it may lead to premature destruction of the cell. This was then followed by change of policy on providing ABO compatible platelet in some institution (Stolla et al., 2015). In 2012, Yazer and coworkers attempted to look on the other adverse reaction of ABO incompatible platelet transfusion by focusing on 162 recipients who develop fever following platelet transfusion. However they have discovered that there is no significant association between ABO incompatibility and FNHTR (Yazer et al., 2012).

1.1.2.2 HLA Class 1 on platelet

HLA Class 1 are very well expressed on platelet, predominantly the HLA-A and HLA-B (Szatkowski et al., 1978) with weak expression of HLA-C (Mueller-Eckhardt et al., 1980). About 2/3 of blood's HLA Class 1 are expressed on platelet and was reported to be of average about 20,000 molecules of HLA class I per platelet (Pereira et al., 1988), however, the amount varies amongst individuals (Kao et al., 1990, Pereira et al., 1988).

Due to the highly polymorphic characteristics of the HLA, immunization to HLA Class 1 can easily occur following transfusion, pregnancy and transplantation. It is reported to be the major cause of immune mediated transfusion refractoriness (McFarland et al., 1997), especially in the multiple transfused patient who need long support of transfusion. In oppose to that, the association of anti-HLA Class 1 antibody in NAIT cases are still debatable (Taaning, 2000). Although some cases of NAIT are probably caused by maternal HLA antibodies reactive with foetal and neonatal platelets, it is difficult to implicate the involvement of anti-HLA Class 1 in the pathogenesis of this disorder because as many as 30% of multiparous women are immunized against HLA class I (Kakaiya et al., 2010, Triulzi et al., 2009), but the majority of them do not give birth to infants with thrombocytopenia.

1.1.2.3 Platelet specific antigen – Human Platelet Antigen (HPA)

Human Platelet Antigen (HPA) are the specific antigen found on platelet residing on glycoprotein complex on the platelet membrane. To date, 33 HPAs have been identified to be expressed on six different platelet glycoprotein (Curtis and McFarland, 2014). These glycoproteins are the GPIIb, GPIIIa, GPIba, GPIbb, GPIa and CD109. The HPA antigens are numbered according to the order of the discovery, with the high frequency antigen is designate as 'a' and the antithetical low frequency antigen is labelled as 'b'. Twelve antigens are being clustered into six biallelic group,

while the other 21 low frequency antigens are designated as 'w' (for workshop) indicating that only antibody only toward the 'b' antigen has been discovered, but not toward its antithetical partner (Curtis and McFarland, 2014). Without the capability to induce the formation of an alloantibody, a genetically determine protein or carbohydrate will not be called as an "antigen", thus these antithetical partner "antigens" are not listed in the list of Human Platelet Antigen.

Whilst some HPAs are commonly implicated in immune-mediated thrombocytopenic disorder (e.g. HPA-1a in NAIT), other HPAs appear to be less immunogenic and therefore rarely implicated (Curtis and McFarland, 2014). Figure 2 below illustrate the platelet glycoprotein (GP) structures, GPIIb/IIIa, GPIa/ IIa, GPIb/IX and CD109, and the locations of HPA antigen they express.

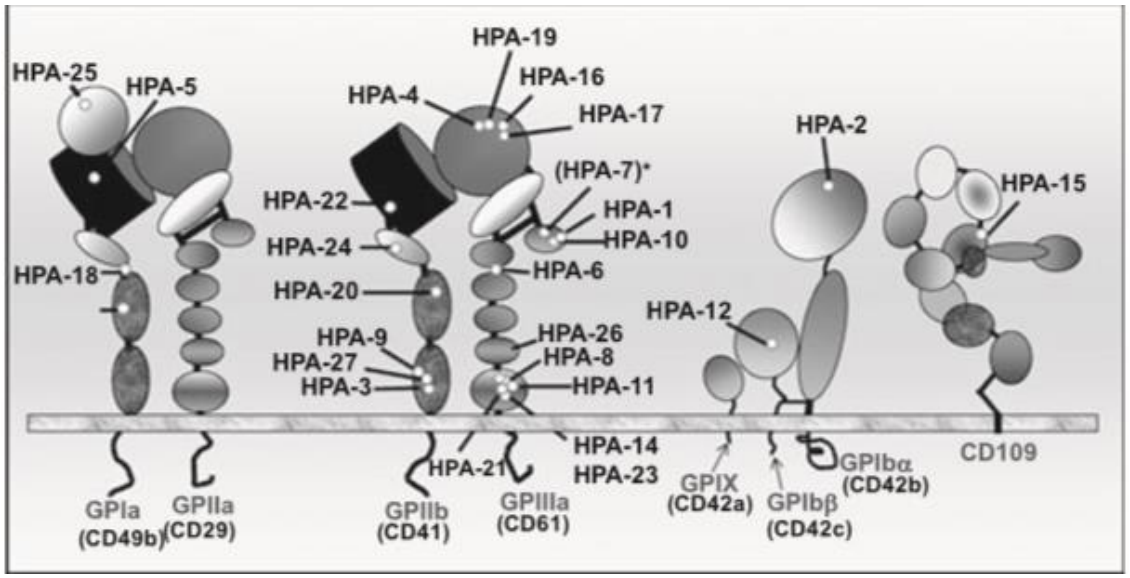


Figure 2 : Platelet glycoprotein (Curtis and McFarland, 2014).

The above illustration depicted the glycoprotein structure of GPIIb/IIIa, GPIa/IIa, GPIb/IX and CD109, with the location of the different human platelet antigen location.

The list of Human Platelet Antigen is tabulated in appendix 1.

1.1.3 Alloimmune thrombocytopenic disorder

Alloimmunization is related to the composition of antigen in the blood product transfused and the immune status of the recipient (Pavenski et al., 2012). Even though platelet alloantigen can be divided into three groups, all alloantibodies toward the antigens are called the platelet reactive antibodies. These antibodies are the causal for platelet clearance by the mechanism known as immune-mediated thrombocytopenia. Disorders caused by the alloimmune-mediated thrombocytopenia is the platelet transfusion purpura (PTP), neonatal alloimmune thrombocytopenia (NAIT), and platelet transfusion refractoriness (PTR).

1.1.3.1 Post Transfusion Purpura (PTP)

Post transfusion purpura (PTP) is a rare acquired disorder that occurs about one to two weeks following a transfusion of blood products (Shtalrid et al., 2006). It is a rare type of delayed adverse reaction triggered by anamnestic immune response (Kaplan, 2002) which eventually lead to immune destruction of platelet leading to severe thrombocytopenia. However the pathogenesis remain unclear whereby the alloantibody of the patient will not only attack the transfused alloantigen-positive platelet, but paradoxically destroy the autologous platelet that is negative for the offending antigen (Shtalrid et al., 2006, Kaplan, 2002). PTP is caused specifically by the HPA group of antigen and in majority of the cases by anti-HPA-1a. However, the occurrence on anti-HPA-1a was observed to be associated with HLA class II allele DRB3*0101. This findings has been observed in majority of the patients with PTP (Kaplan, 2002, McFarland, 2001).

PTP incidence were reported to be 1 in 50,000 – 100,000 transfusion (Metcalfe, 2004) and were found more in middle age female rather than man. The simple explanation was due to the previous sensitization by multiple pregnancy (Shtalrid et al., 2006). However, in an 11 years of retrospective study done by Shtalrid and colleagues in 2006 from their working hospital, the incidence were found to be 1 : 24,000 transfusion, implicated in 4 women and two men. The big difference in

incidence is maybe due to the potential misdiagnosis of PTP since the critically ill patient usually have alternative explanation for thrombocytopenia (Shtalrid et al., 2006) and the lack of confirmatory laboratory data to rule out the alternative reason for the low platelet count (Roubinian and Leavitt, 2015). Furthermore, the distribution of HPA-1bb phenotype is also a factor that stimulate the acquisition of this disorder. In a cohort study done by (Menis et al., 2015), they found out that there is no association of sex with incidence of PTP. Since PTP is primarily due to the previous exposure of platelet alloantigen, men and women with previous history of transfusion is also at risk. Therefore sex should not be a factor for suspecting a PTP (Roubinian and Leavitt, 2015). Since PTP can destruct the allogenic platelet as well as autologous, platelet transfusion may not be beneficial. Administration with IVIG is the preferred therapy (Delicou and Bellia, 2015).

1.1.3.2 Neonatal alloimmune thrombocytopenia (NAIT)

NAIT refers to a disorder that affecting foetal and neonatal. It happens when there is a fetomaternal incompatibility and is almost similar to the haemolytic disease of newborn (HDN). However, in NAIT, platelet is the target cell of destruction (Taaning, 2000), and it may occur in first pregnancy (Sachs, 2013, Carr et al., 2015). NAIT happens when the foetus inherited platelet antigen from the father which absent on the mother's platelet. Upon sensitization, mother will produce IgG platelet alloantibody and this antibody are capable to travel through the placenta, recognizing the specific platelet on the foetal antigen thus destroy it. The clearance of the antibody-coated platelet will lead to low platelet count thus increasing the chance of spontaneous bleeding in utero or after delivery. The symptom can vary from asymptomatic through incidental findings of thrombocytopenic condition to severe life-threatening situation such as intracranial haemorrhage (Delicou and Bellia, 2015). Platelet production in the fetus begin around 5 weeks after conception and reach normal postnatal range of 150 – 450 x 10^9/L by the end of second trimester (Carr et al., 2015), with major antigens are very well expressed at week 19 of gestation (Delicou and Bellia, 2015).

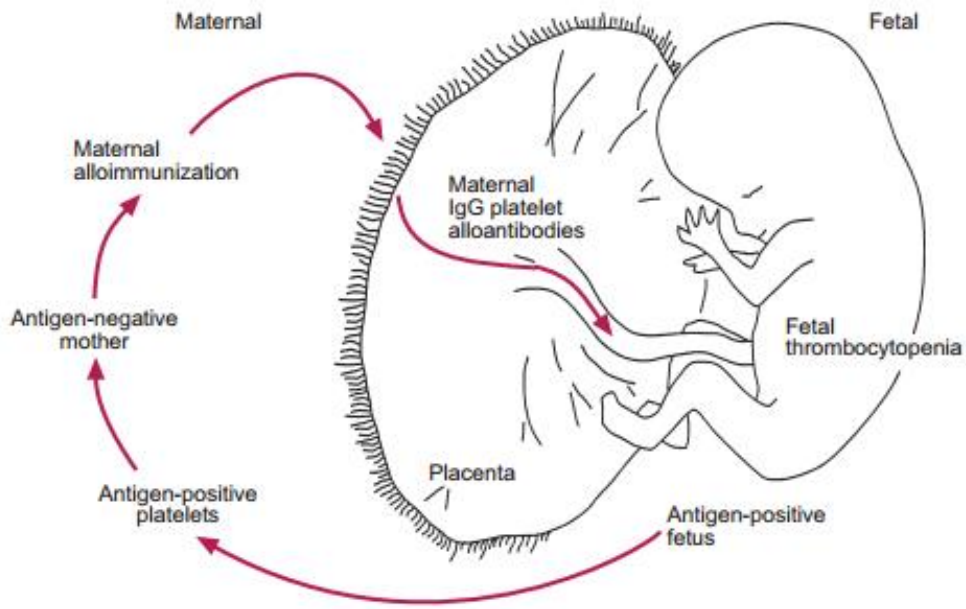


Figure 3 : Pathophysiology of neonatal alloimmune thrombocytopenia (NAIT) (Blanchette et al., 2000).

In Caucasian, the incidence of NAIT was reported to be about 1 in 1000 deliveries (Bakchoul et al., 2014). It has also been reported to be occurring in the Malaysian population (Armawai et al., 2014) in much smaller numbers. In Caucasians, about 75% NAIT cases are induced by anti-HPA-1a on GPIIIa, and another 15% of cases are caused by anti- HPA-5b on GPIIa (Sachs, 2013). Conversely, in Asian, predominantly in Japanese, the most frequently detected antibodies in NAIT are anti-HPA-4b and anti-HPA-5b (Kiyokawa et al., 2014). Although NAIT has been reported to be exclusively associated with the HPA antibodies, there are reports of anti-HLA associated with NAIT, but the evidence has been indirect and weak. Therefore, the correlation of NAIT and anti-HLA Class 1 remain a myth (Taaning, 2000). Nevertheless, HLA Class II determinants is said to have an association with HPA-1a alloimmunisation. Similarly as in PTP, maternal immunization against HPA-1a is genetically linked to the presence of the HLA class II allele DRB3*01:01. Approximately 90% of immunized women are DRB3*01:01 positive (Sachs, 2013). Another report by Delicou and

Bellia also show that HPA-1a-negative women who have become sensitised to the HPA-1a antigen also possess the HLA-B8, HLA-DR3, and DR52a antigens. This lead to assumption that these alleles are markers for increase risk of alloimmunisation (Delicou and Bellia, 2015).

Thrombocytopenia and bleeding in neonate is the common sign of NAIT. An extensive study done by Mueller-Eckhardt et al., 1989a shows that the most common bleeding signs is petechiae, haematoma and followed by melena (bloody stool). Haemoptysis, retinal bleeding and haematuria was also seen in some cases. These NAIT babies also were commonly observed to have low birth weight (Sachs, 2013). Although thrombocytopenia is often temporary and resolves spontaneously, it can be severe with serious bleeding including intracranial haemorrhage (Bakchoul et al., 2014). This alarming condition may resulted in persistent neurologic impairment and death (Blanchette et al., 2000). Therefore, rapid management is needed with the primary goal is to stop or prevent bleeding from occurring, more importantly to prevent intracranial haemorrhage. Neonate should be transfused with negative-antigen platelet type to rapidly increase the platelet level count to 50 x 10^9/L. In situation where typed platelets are not available from random donor, compatible platelet can be obtain from the mother via platelet apheresis donation. Prior to transfusion, platelet from mother must be washed to remove maternal plasma that contain the antibody which could prolong the thrombocytopenia. It also must be irradiated to prevent graft-versus-host-disease (GVHD) (Carr et al., 2015). High-dose intravenous immununoglobulin may also be used to prolong the survival of the autologous platelet (Bussel JB, 2009, C, 2008, Birchall JE, 2003). In 2014, a study was done by Bakchoul and colleague, on the effect of IVIG that was administered together with random platelet transfusion. However, they did not observe any additional benefit from it.

1.1.3.3 Platelet Transfusion Refractoriness (PTR)

Refractoriness to platelet transfusion refers to the lack of response, by specifically looking at the post-transfusion count increment (Pavenski et al., 2012), after 2 consecutive platelet transfusion of adequate allogeneic platelet dose (Delaflor-Weiss and Mintz, 2000) of fresh, random donor ABO compatible platelets (Rebulla, 2005). Platelet refractoriness will result in a persistent thrombocytopenic condition due to the shortened survival of transfused platelet in the recipient's blood circulation. To determine the efficacy of the platelet transfusion, post-transfusion count can be determined from two calculation formula; (1) corrected count increment (CCI) in relating it to the patient's body surface area or (2) percent platelet recovery (PPR) in the context of estimated blood volume of the patient (Wood et al., 2005, Delaflor-Weiss and Mintz, 2000, Davis et al., 1999). Each formula compares the incremental change of platelet count from the initial in relative to the dose of platelet transfused in the first hour, and after 18 to 24 hours. The calculation formula are as in table 1.1

Table 1.1 : Calculation formula for measurement of platelet transfusion efficacy and value suggestive of refractoriness

Measures of transfusion outcome	Formula	Value suggestive of refractoriness
Corrected count increment (CCI)	$\frac{\text{ACI x body surface area } (m^2)}{\text{No of platelet transfused x } 10^{11}}$	At 10 – 60 min, CCI <7,500/uL At 18 – 24 hrs, CCI <5,000/uL
Percent platelet recovery (PPR)	$\frac{\text{ACI x total blood volume x 100\%}}{\text{No of platelet transfused x } 10^{11}}$	At 10 – 60 min, PPR <20 % At 18 – 24 hrs, PPR < 10%

Note : ACI (Absolute count increment) = (post-transfusion count/uL – pre-transfusion count/uL (Pavenski et al., 2012)

Platelet refractoriness can be immune or non-immune cause (Pavenski et al., 2012, Delaflor-Weiss and Mintz, 2000). The non-immune cause can be due to the platelet product itself, such as poor platelet quality and old aged platelet (Delaflor-Weiss and Mintz, 2000) or due to the clinical condition of the patient that accelerated with platelet consumption such as splenomegaly, disseminated intravascular coagulopathy (DIC), bone marrow transplant and graft versus host disease (GVHD) (Ishida et al., 1998, Bishop et al., 1991, Slichter, 1990, Bishop et al., 1988). It contributed to more than 80% of all refractory cases. Conversely, immune-mediated platelet refractory is only about 20% of all the refractory cases, with majority of it is due to the alloimmunization of HLA, followed by HPA, or both HLA and HPA (Pavenski et al., 2012). The ABH antibodies also have been reported to cause immune refractoriness (Hod and Schwartz, 2008, Laundy et al., 2004) especially in the high expresser platelet. These antibodies that directed toward the specific antigen of HLA, HPA and ABH are called platelet reactive antibodies and is the cause for accelerated platelet destruction and transfusion failure (Agarwal et al., 2014). PTR is commonly associated with multiple platelet transfusion. Despite of receiving multiple episode of platelet transfusion, high number of haematological patient were reported to develop immune refractory to platelet transfusion even after receiving single episode of multiple RBC transfusion (Rebulla, 2005).

The 1-hour CCI or PPR count may help in establishing the cause of refractoriness. Commonly, there will be no increment in the 1-hour count for immune refractory, as compared to the non-immune cause where it will be normal during the 1-hour count but reduced at 18-24 hours count (Craig H. Fletcher, 2015, Delaflor-Weiss and Mintz, 2000), with exception to splenomegaly. In platelet refractory management, evaluation of the clinical condition refractory pattern is vital to discriminate the type of refractoriness. While management approach of non-immune cause is simply by treating the underlying cause, managing patients with immune-mediated platelet refractoriness can be very challenging (Lee and Ayob, 2015).

1.1.4 Support for the platelet refractoriness patient

When a patient was discovered for persistent thrombocytopenia following two good quality of platelet transfusion, he will be further evaluated to identify the source of refractory. While the management of non-immune platelet refractoriness is mainly depending on treating the underlying illness (Stolla et al., 2015), the immune platelet refractoriness is usually managed by transfusion of matched platelets (Pavenski et al., 2012).

1.1.4.1 Antigen-negative, HLA matched and epitope matched

Through an antibody identification test, offending antibody type that cause refractoriness to the patient can be identified. Once it has been identified, a negative-antigen-platelet toward the corresponding antibody will be search from the inventory stock by phenotyping process or by checking through the donor registry from the donor pool. In this scenario, patient genotyping is not really necessary, and platelet can be issue without crossmatch. Conversely, in patient that is highly sensitized with multiple antibody, or need long term platelet support, the patient's sample will be tested for HLA typing. If the refractory is due to anti-HPA, HPA typing will also be performed. While waiting for the result that could take for days, ABO compatible fresh platelet is given to support the thrombocytopenic condition (Wiita and Nambiar, 2012). To supply platelet based on this HLA match approach, the donor and patient must be identical for ABO group and all four locus of HLA-A and HLA-B or BU (level of mismatch) match grade where the donor and recipient are identical at only two or three locus. If the refractory is due to HPA, the donor and recipient must be identical for HPA too (Lee and Ayob, 2015, Kopko et al., 2015).

Another methodology of finding a match platelet for HLA-refractory patient is through epitope-based matching. Epitope are composed of a short linear sequence or discontinuous amino acid residues that form a cluster on a molecular surface (Duquesnoy and Askar, 2007). Patient will only

make antibodies to foreign epitopes. Thus, instead of matching for full HLA type, matching of epitope can be done by using a "HLA-matchmaker" software.

Through any of these methodologies, the ideal platelet donor that have a match-HLA or minor mismatch-HLA will be called upon to donate and the platelet will be issued to the specific patient (Silva et al., 2010, Bub et al., 2013). However, the real challenges are due to the HLA type itself. HLA is very polymorphic as compared to HPA, thus defies the management of the refractory patient through these approaches. The HLA-typed donor pool must be large enough to increase the chance of finding an ideal match (Petz et al., 2000) but the platelet may not necessary be crossmatched prior to issuing it to the patient. Once donor is identified through the registry, donor will be called upon for donation. Even it seems easy, this may take few days for the donor to turn up to the blood centre and donate.

1.1.4.2 Platelet crossmatch

Crossmatch is theoretically more sensitive than the HLA match owing to its capability to detect any platelet reactive antibodies, including the anti-glycoprotein which can be found in the autoimmune thrombocytopenic disorder. While HLA matching approach is time consuming, crossmatch can be done in few hours upon receiving the patient's serum. It provide rapid supply of compatible blood and often the choice of a blood centre that don't have large registry of typed HLA profile. Furthermore, crossmatch can be done with ABO-compatible blood, and platelet can be supplied before completing the HLA antibody and HLA genotyping of the patient. However, this test is suitable for patient with low to moderate immunization to HLA Class 1 antibody. In patient who is highly sensitized to the HLA antigens, crossmatch will not be easy, as it will be difficult to find a compatible unit (Kopko et al., 2015).

Platelet crossmatch can be performed either by solid phase red cell adherence (SPRCA) assay or by flow cytometry. It is crucial that in crossmatching, any incompatibility due to platelet reactive antibody including the ABH, HPA and HLA system can be detected. In SPRCA assay, the intact platelet from the donor is bound to the U-shape microplate well by a coating monoclonal antibodies. Patient serum is added in and incubated to allow for antigen-antibody interaction. When the incubation is over, the microplate well is washed to remove any unbound antibody, followed by adding an indicator RBC that is coated with anti-human globulin (AHG). Sample is then centrifuged and result will be analysed. If the patient sample contain any platelet reactive antibody, the red cell indicator will form a monolayer spread over the well, however, if the reaction is negative, the indicator red cell will be pelleted at the bottom of the well.

SPRCA-based-assay is also commercially available. Capture-P® by IMMUCOR, USA has been used by the blood centres of the pacific reference laboratory in San Francisco, USA (Wiita and Nambiar, 2012), while MASPAT, Sanquin Blood Supply, Amsterdam, are the choice of many researchers for crossmatching (Salama et al., 2014, Schallmoser et al., 2006, Jia et al., 2014).

Another approach for crossmatching is by using flow cytometry assay. The test is called platelet immunofluorescence testing or PIFT. In PIFT, intact donor platelet is incubated with patient serum. After incubation, the tube are washed to remove any unbound antibodies, followed by adding a fluorescence-labelled-anti human IgG (FITC-IgG) and incubated again for the reaction to take place. Next, the mixture is washed again to remove the excess unbound FITC-IgG and the result will be ready to be analysed by flow cytometer.

The principle and result interpretation of PIFT and SPRCA will be illustrated in figure 12 until figure 15 at page 60 and 63.

Crossmatch-compatible platelets are presumed to be lacking the antigen which the antibody present, while incompatible crossmatch is presumed to possess the antigen in which reacting with the clinically significant antibody in the patient. The incompatible result will predict a poor response in over 90% of transfusion. Conversely, a compatible crossmatch in an in vitro testing does not guarantee good survival of the transfused platelet in vivo. Compatible crossmatch is predictive to be successful in about 50-60% of the transfusion (Marwaha and Sharma, 2009). While it does not promise an improved platelet count, crossmatch does improve on the availability of the platelet for the patients where platelet can be obtain without the need to do HLA and HPA typing to the patient. Donor and many platelet can be crossmatch simultaneously in a rapid time frame (Craig H. Fletcher, 2015).

Each methodologies on supplying platelet to the immune-mediated thrombocytopenic patient have its own strength and weakness, hence, single approach might not be good enough. Depending on the clinical diagnosis of the patient, some large blood transfusion service may combine all approach ; antigen-negative, HLA-matching and crossmatch in order to provide the best platelet product to the immune-mediated thrombocytopenic patient (Marwaha and Sharma, 2009).

1.2 Problem statement

Various strategies have been developed in order to support platelet crossmatch for alloimmune-mediated thrombocytopenic patient. Some approaches have been described in paragraph 1.1.4. Other therapies include the drug usage for immunosuppression, rituximab, intravenous immunoglobulin (IVIG), plasma exchange and immunoadsorption have also been tried but with little success (Agarwal et al., 2014). In Malaysia, the management of thrombocytopenic patients who are refractory to random platelet transfusion continues to be in dilemma. National Blood Centre (NBC) is the only institution that offers the platelet antibody testing in the country, hence, it is the only centre that is capable of doing the platelet crossmatch test.

While it sound simple, the setback of the platelet crossmatch procedure was actually the choice of the method itself. There are two different categories of testing. Glycoprotein-capture-based assay is a very sensitive and specific assay. However, it is not suitable for crossmatching because it test specifically on the glycoprotein of interest, and way too expensive. The example of this type of assay is the monoclonal alloantibody immobilization of platelet antigen (MAIPA). The other group of method is the intact-platelet-based assay, which will detect the antibody toward antigens presented on the platelet surface, including the ABH antigens. Therefore it is more suitable for the application in crossmatching. The example of this type of assay are the Solid Phase Red Cell Adherence Assay (SPRCA) and Platelet Immunofluorescence Assay (PIFT).

In NBC, platelet immunology testing are done by both MAIPA and SPRCA assay. However, due to the principle of intact-platelet-based-assay, SPRCA is the more suitable method for platelet crossmatching process. It is a procedure that requires manual preparation of in-house reagents, manual testing, manual interpretation and manual result documentation (not computer-generated-evidence-based result). This fully manual processes may give chance to bias as

competency of operator becomes an important confounding factor. Furthermore, SPRCA assay must be tested in batch due to the protocol that utilize microtiterplate with coated capture antibody on the base of the microplate well. The maximum crossmatch per batch can be up to 45 crossmatches. Even though it can be used for a much smaller numbers, single crossmatch seems impractical when relating it to cost, laboriousness, and time taken to complete the test. Therefore, due to the difficulties and nature of test that requires highly technical skilled personnel, this test is only offered at the NBC, where staff performance being supervised and competency is regularly assessed and monitored.

Eventhough SPRCA works well as a crossmatch protocol, the biggest limitation of SPRCA is that it is not suitable for single crossmatching. Single crossmatch is important when it comes to checking the compatibility of the patient with a specific and single apheresis donor. As the cost of the apheresis product is very expensive and continue to increase each year, it is better to ensure that the plateletpheresis unit is compatible toward the immune-mediated thrombocytopenic recipient in order to avoid wastage and unbeneficial transfusion. Apart from that, single donor platelet transfusion may reduce the risk of septic transfusion reaction (Ness *et al*., 2001). Hence, by looking into all the limitations of the current available method, and by considering the need for intensive medical support in terms of transfusing compatible platelet to the alloimmune-mediated thrombocytopenic patient, there is an essential need to search for an enhance protocol that can overcome the limitation of SPRCA. Moreover, the new method should be feasible to be adopted by other blood transfusion centre in this country. Therefore, this study is conducted to evaluate the qualitative value and feasibility of PIFT as a tool for platelet crossmatch testing, in order to heighten the service for provision of compatible platelet supply for the immune-mediated thrombocytopenic patient in Malaysia.

1.3 Research Objectives

1.3.1 General objectives

To evaluate the qualitative performance of PIFT for platelet crossmatching and analyse the feasibility of the assay as a tool for the provision of compatible platelet supply to the immune-mediated thrombocytopenic patient.

1.3.2 Specific objectives

1.3.2.1 To optimize PIFT by evaluating the most optimal FITC-IgG dilution that work best in PIFT

1.3.2.2 To measure the sensitivity, specificity, result predictive value and false positivity rate of the PIFT assay.

1.4 Research question

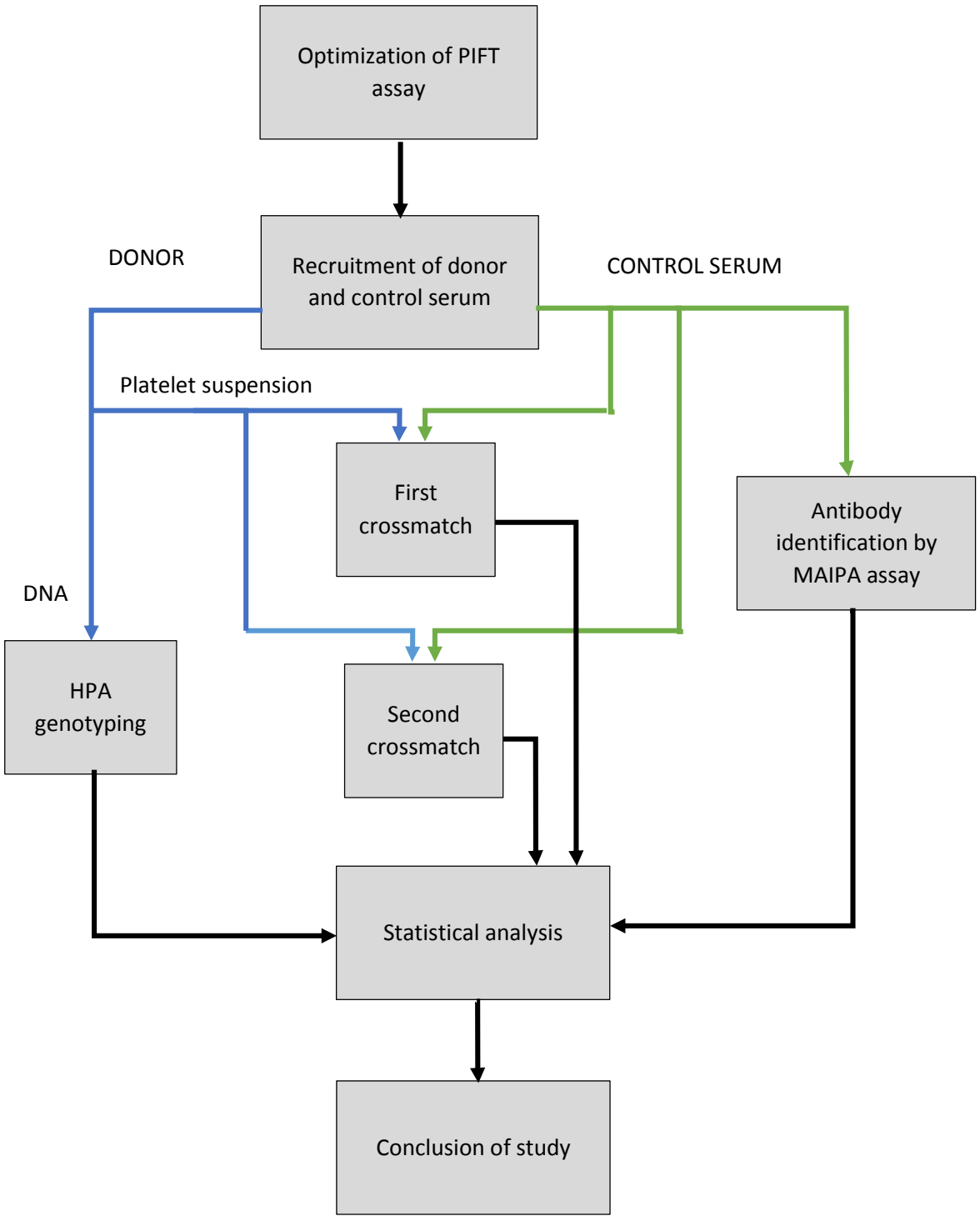
Does PIFT have a good qualitative performance for platelet crossmatch test, can it overcome the limitation of SPRCA and is it feasible to be implemented in the blood bank for platelet crossmatch service?

1.5 Hypothesis

H_O : PIFT assay is a depreciate protocol with poor qualitative value, and it is not feasible to be implemented for platelet crossmatch service

H_A : PIFT assay is an enhance protocol with good qualitative value and it is feasible to be implemented for platelet crossmatch service

1.6 Flowchart



Key :

Blue line : donor's sample

Green line : control serum

Black line : result

Chapter 2

MATERIALS AND METHODOLOGY

2.1 Introduction

"Platelet Crossmatch By Flow Cytometry : Evaluation and Feasibility Study" is an applied research in searching for an enhanced protocol for blood bank service application. This research is carried out in National Blood Centre (NBC) specifically in the Platelet and Haematology Laboratory. PIFT protocol was adopted from the National Institute for Biological Standards and Control (NIBSC) website http://www.nibsc.org/science_and_research/biotherapeutics/platelets.aspx. The assay was then optimized in local laboratory setting, in order to establish the best FITC-IgG dilution by using known remaining serums donated by the Platelet Laboratory, NBC. Once PIFT has successfully optimized, qualitative evaluation were then carried out.

In this qualitative study, 20 voluntary donors were randomly selected. These donors were genotyped for their HPA profile. Platelet crossmatch is done on batch of control serum that have either HPA-antibodies with known specificity, HLA-antibodies with unknown specificity, serum with no antibodies, and serum with specificity toward the glycoprotein itself (autoantibody). The result of the crossmatch will then be compared to the HPA genotype profile of the donor that will serve as a base to infer a true positive and true negative result. However, for control serum with HLA-antibody, the result can only be interpreted for absolute compatibility result, but not for qualitative analysis because the information on the antibody specificity is not obtainable. This part will be elaborated in Chapter 4 – limitation of study.

In this study, reagents are commercially purchased, or otherwise stated. The details of the reagents are listed in appendix 2.

2.2 Platelet donor recruitment

20 donors were involved in this study. Sampling were made randomly from group O RhD positive voluntary platelet apheresis donor that donates at Pusat Darah Negara, Kuala Lumpur, *via* walk-in donation. Samples were collected from 25th February 2015 to 17th March 2015 and informed consent was obtained. All donors were given a unique identification number, known as "Panel ID" for the purpose of this study. Ethical clearance have been granted from National Medical Research Register (NMRR) of Ministry of Health (MOH). Reference number is NMRR-14-1293-23185 (IIR).

2.2.1 Inclusion and exclusion criteria

Platelet apheresis donor were recruited from donors that are eligible to donate at the time of donation. The eligibility of donation are as described in Transfusion Practice Guidelines for Laboratory Personnel, 3rd edition, 2008. All donors were screened for HIV, Hepatitis B, Hepatitis C and Syphilis with serology screening and nucleic acid testing to ensure that the blood product and blood samples is free from pathogen that could jeopardize the safety of individual that may be in contact with the sample.

2.2.2 Blood sampling process

Venous blood samples were collected from donor's vein before the apheresis donation commence. Blood were drawn into 6ml K2.EDTA tube without gel, and mixed gently to ensure that the anticoagulant homogenized with the blood to avoid clotting. All tubes were then labelled with Panel ID for identification of sample and were sent to the Platelet Laboratory. There, the sample is further processed for DNA extraction as described in paragraph 2.2.3, and for preparation of platelet suspension as described in paragraph 2.5.3 at page 33.

2.3 Platelet Donor - DNA extraction

DNA extraction was performed by using commercial DNA extraction kit (Exgene™ Blood SV Mini, GeneAll®, South Korea). All reagents, buffers (BW, TW and AE) and spin column used in this process are part of the kit, with exception to the absolute ethanol and microtubes.

To start the DNA extraction process, the donor's whole blood sample from 2.2.2 was centrifuge at 4,000g for 10 minutes. After centrifuging, 3 distinctive layers of plasma, buffy coat and pack cells can be easily identified. 200ul of blood sample which contain large amount of the buffy coat layer was transferred to a 1.8ml-sized microtube, follow by the addition of 20ul of Proteinase K. The mixture was gently vortex to mix the solution. Next, 200ul of BL buffer was added into the tube, followed by a vigorous vortex until the colour of the sample change to black-brown which indicate cell lysis. Sample was then incubated at 56°C for 10 minutes.

After incubation, 200ul of absolute ethanol was added to mixture followed by a strong vortex. Clot may form at this phase. The content of the microtube was then carefully transferred to a spin column with attempt to avoid transferring the clot as it may clogged the spin column. The spin column was then centrifuged at 13,000rpm for 3 minutes. Once the centrifuging stop, the flow-through is discarded and the spin column is inserted into a new-clean collection tube. 500ul of BW buffer is added into the same spin column and was centrifuged again at 13,000rpm for another 3 minutes. Once the centrifuging stop, the clarity of the flow-through and spin column is examine by visual inspection. If there are still residual of brownish colour remain, repeat the washing with BW buffer until clear, and proceed to the next step with a new-clean collection tube.

Next, 700ul of TW buffer was added into the spin column, followed by centrifugation of the spin column at 13,000rpm for 3 minutes. The flow-through was discarded and the same collection tube was re-inserted back into the spin column, followed by another centrifugation for 3 min at