

Platelet antibody screening by flow cytometry is more sensitive than solid phase red cell adherence assay and lymphocytotoxicity technique: a comparative study in Thai patients

Jarin Buakaew¹, and Charuporn Promwong¹

Summary

The objective of this study was to compare the sensitivity and specificity of lymphocytotoxicity test (LCT), solid phase red cell adherence assay (SPRCA) and flow cytometry in detecting platelet reactive antibodies against human leukocyte antigens (HLA) class I and human platelet antigens (HPA). Sera from 38 thrombocytopenic patients and 5 mothers of thrombocytopenic newborns were screened for platelet reactive antibodies by these three methods using screening platelets and/or lymphocytes panels derived from six subjects. The sensitivity and specificity of each method and levels of agreement were analysed. HLA antibodies were found in 18, 17 and 19 out of 43 patients' sera tested by LCT, SPRCA and flow cytometry, respectively. Four out of 43 patients' sera were reactive against HPA by flow cytometry, but were reactive to only 2 sera by SPRCA. Using flow cytometry as the reference method, the sensitivities/specificities of SPRCA and LCT in HLA antibody detection were 84.21/95.83% and 94.73/100%, respectively, with a good strength of agreement. SPRCA had 50% sensitivity and 100% specificity in HPA antibody detection compare to flow cytometry. Flow cytometry

appeared to be the most sensitive technique compared with SPRCA and LCT for both HPA and HLA antibody screening. SPRCA sensitivity was too low for HPA antibody detection, but this might be because of the small number of samples. There was one serum from the mother of a baby suffering neonatal alloimmune thrombocytopenia (NAIT), in whom SPRCA could not detect HPA antibodies, while flow cytometry came out positive. Therefore, SPRCA should not be used in NAIT investigation and flow cytometry should be employed instead. (*Asian Pac J Allergy Immunol 2010;28:177-84*)

Key words: platelet reactive antibodies, sensitivity/specificity, solid phase red cell adherence assay, flow cytometry, lymphocytotoxicity test.

Introduction

Platelet alloantibodies can cause immune-mediated platelet destruction because a variety of platelet-reactive antibodies react with antigens on platelet membranes such as human leukocyte antigen (HLA) class I molecules and human platelet antigen (HPA)¹⁻⁴. Platelet refractoriness, neonatal alloimmune thrombocytopenia (NAIT) and post transfusion purpura (PTP) are examples of disorders caused by platelet alloantibodies⁴⁻⁷. Several platelet reactive antibody detection techniques have been reported, such as lymphocytotoxicity test (LCT), solid phase red cell adherence assay (SPRCA), enzyme-linked immunosorbent assay (ELISA), monoclonal antibody-specific immobilization of platelet antigens (MAIPA) and flow cytometry⁸⁻¹². Each technique may have some limitations in routine practice such as complicated procedures, highly skilled personnel needed, high cost and low sensitivity.

From the ¹ Blood Bank and Transfusion Medicine, Department of Pathology, Faculty of Medicine, Prince of Songkla University, Hat Yai, Songkhla, Thailand
Corresponding author: Charuporn Promwong,
Address: Blood Bank and Transfusion Medicine Unit, Department of Pathology, Faculty of Medicine, Prince of Songkla University, Hat Yai, Songkhla 90110, Thailand; e-mail: pcharupo@hotmail.com



In Thailand, HLA antibodies are commonly detected by the LCT technique, e.g., HLA antibody screening in patients with platelet refractoriness and potential renal transplant recipients. When HPA antibodies are suspected, SPRCA is commonly used. In our laboratory, the LCT and SPRCA techniques were previously used to investigate HLA and HPA alloantibodies, but the results were unsatisfactory. Therefore, the flow cytometry technique was developed and the sensitivity and specificity comparisons with the previous methods were carried out. The objective of this study was to determine the sensitivity and specificity of flow cytometry technique for HLA and HPA antibody detection compared to those of LCT and SPRCA.

Methods

Patients

The study was carried out during January 2005 to October 2008. Ten healthy control subjects, 38 thrombocytopenic patients and 5 mothers of babies suffering NAIT were recruited. The sera were stored at -70°C until they were used. They were tested for platelet reactive antibodies in parallel by LCT, SPRCA and flow cytometry techniques against panels of screening target cells derived from six random, group O blood donors. A panel of six mononuclear screen cells was used in LCT and a panel of six platelets was used in SPRCA and flow cytometry. The study was approved by the Ethics Committee, Faculty of Medicine, Prince of Songkla University.

Untreated and chloroquine-treated platelet panel preparation

Five milliliters of whole blood in acid citrate dextrose (ACD) were obtained from each donor and centrifuged at $500 \times g$ for 10 minutes and then platelet-rich plasma (PRP) was collected. A panel of six platelets for SPRCA was prepared as follows: PRP was frozen at -70°C for 1 hour or more and then washed twice with 0.2% BSA/0.9% NaCl solution (washing solution) by centrifugation at $1,000 \times g$ for 4 minutes. The platelet pellet was treated with 1% paraformaldehyde for 5 minutes then washed twice with washing solution and its density was adjusted to approximately $1 \times 10^8 \text{ mL}^{-1}$ and used as an untreated platelet target panel.

A panel of six platelets for the flow cytometry study was prepared and the freshly separated PRP was immediately washed. The platelet pellet was

treated with 1% paraformaldehyde for 5 minutes and then the untreated target platelet suspension was prepared as described above.

A panel of six chloroquine-treated platelets for the HPA antibody study was prepared according to Lown *et al.*¹¹. HLA antigens were removed from platelet surfaces by chloroquine treatment, but HPA antigens remained. Two millilitres of a 0.2 M chloroquine disulphate solution, at pH 4.0, was added to the 100 μL of untreated platelet suspension. The mixture was incubated for 30 minutes at room temperature and then washed three times with washing solution by centrifugation. The platelet pellet was fixed with 1% paraformaldehyde for 5 minutes and then washed twice with washing solution. The platelet suspension density was adjusted to approximately $1 \times 10^8 \text{ mL}^{-1}$.

Lymphocytotoxicity test (LCT)

LCT was performed according to Levin *et al.*⁹ using the method originally described by Terasaki *et al.*¹². Five milliliters of ACD whole blood were collected from each donor and then mixed with 2.5 mL of PBS and underlayered with 2.5 mL of Lymphoprep (Nyegard, Norway). The sample tubes were then centrifuged at $1,000 \times g$ for 15 minutes and the mononuclear cell interfaces were harvested and washed twice with PBS. Contaminated platelets were eliminated by precipitation with 50 μL of bovine thrombin and then the mononuclear cell densities were adjusted to $1 \times 10^6 \text{ mL}^{-1}$ and their viability was estimated by trypan blue. One microliter of each patient's serum and positive and negative controls were incubated with 1 μL of mononuclear panel cells for 30 minutes at room temperature in Terasaki trays and 5 μL of rabbit complement (One Lambda Inc, USA) was added to each well and incubated for 60 minutes, 5 μL of eosin were added and left for 2 minutes before 10 μL of formaldehyde were added. The tray was covered and left at least four hours at room temperature before reactions were read. The reactions against the screening cells were considered positive when dead cells in each well were more than 50% of the total. When two or more of the six mononuclear cells in the panel showed a positive reaction, the patient was considered to be immunised against a broad range of HLA antigens⁹.



Solid phase red cell adherence assay (SPRCA)

SPRCA was performed according to the protocol from the National Blood Centre, Thai Red Cross Society and Lown *et al*^{11,13} using the method originally described by Shibata *et al*¹⁴. Fifty microliters of a 20 µg.mL⁻¹ concentration of rabbit antihuman thrombocyte solution (DakoCytomation, Denmark) in a pH 10 carbonate buffer were coated to a round bottomed microplate (NUNC, Denmark) and incubated for 2 hours at 22 °C. The buffer was then discarded by rinsing the coated microplate wells with 0.2% BSA/PBS. A platelet monolayer was prepared by adding 50 µL of platelet suspension, either chloroquine-treated or untreated platelets into the microplate wells. The microplate was centrifuged at 1,700 rpm for 2 minutes using a microplate centrifuge (Kokusan, Japan) and, after discarding the supernatant, the microplate was incubated at 37 °C for 15 minutes. Non-adherent platelets were washed off with 0.2% BSA/PBS six times. 100 µL of 1.9% glycine solution and 50 µL of test sera were added to the platelet monolayer in the microplate wells and incubated at 37 °C for 15 minutes. After incubation, unbound immunoglobulins were removed by washing the microplate wells with 0.2% BSA/PBS six times. Fifty microliters of 0.4% suspension of group O Rh(D) positive red cells, sensitized with anti-D antibodies (indicator cells), together with 50 µL of anti-human IgG (dilution 1:4) were added. The microplate was gently mixed and centrifuged at 2,700 rpm for 2 minutes. The reactions were examined for either effacement (a reactive or positive reaction result) or a button of agglutinated red cells (non-reactive or negative reaction result). When two or more out of the six target platelets in the panel showed positive reaction, the patient was considered immunised against HLA class I or HPA antigens¹⁵.

Flow cytometry technique

The indirect immunofluorescence test by flow cytometry was performed with some modification according to the protocol from the National Institute for Biological Standards and Control (NIBSC) of the UK¹⁶ using the method originally described by Kiefel *et al.* and Kohler *et al.*¹⁷⁻¹⁹. Fifty microliters of each platelet suspension (chloroquine-treated and untreated platelets) were incubated with 50 µL of test serum at 37 °C for 30 minutes and washed three times by centrifugation at 1,000 X g for 4 minutes. Platelet suspensions

were incubated with 50 µL of fluorescein isothiocyanate (FITC)-conjugated F(ab')₂ fragment goat anti-human IgG (dilution 1:50) and 5 µL of phycoerythrin(PE) conjugated anti-CD41 (Immunotech, Beckman Coulter, France) at room temperature for 30 minutes in the dark. After washing with 0.2% BSA/PBS/EDTA, the supernatant was discarded and the platelet pellets were resuspended in 0.2% BSA/PBS/EDTA and then the flow cytometric analysis was performed.

Stained cells were analysed by flow cytometry (Cytomics FC500, Beckman Coulter, USA) and approximately 10,000 cells per test sample were counted. The relative fluorescence intensity was expressed as a mean channel number (MCN), where a MCN greater than 25% was considered positive for platelet-reactive antibodies. When two or more of the six platelet wells in a panel showed positive reactions, the patient was considered immunised against HLA class I or HPA antigens¹⁵.

Data analysis

The sensitivity and specificity of the techniques were compared with the reference assay. The following definitions of sensitivity and specificity were used.

$$\text{- Sensitivity} = \frac{\text{true positive sera}}{\text{true positive} + \text{false negative sera}} \times 100$$

$$\text{- Specificity} = \frac{\text{true negative sera}}{\text{true negative} + \text{false positive sera}} \times 100$$

The true positive referred to the number of positive sera in both the test and reference methods, whereas the true negative indicated the number of negative sera in both methods. The false positive represented the number of sera that showed a positive result in the test method but a negative result in the reference method and the false negative corresponded to the number of sera that showed a negative result only in the test method¹⁹.

Statistical analysis

The result of each test was classified as either negative or positive. For every combination of two techniques, a 2x2 table was generated and the Pearson's chi-square test was performed to check for independence between the two tests. All p values were two-sided, and p values less than or



Table 1. Results of HLA antibody screening using lymphocytotoxicity test (LCT), solid phase red cell adherence assay (SPRCA) and flow cytometry assay (Flow) in 43 patients.

Patients' diseases	Reactive samples			Non-reactive samples		
	Flow	SPRCA	LCT	Flow	SPRCA	LCT
1. Haematological malignancy with platelet refractoriness (n=21)						
-Concordance	14	14	14	5	5	5
-Discrepancy	1 0	0 1	1 0	0 1	1 0	0 1
2. Idiopathic thrombocytopenic purpura (n=13)	1	0	0	12	13	13
3. Mothers of babies with NAIT (n=5)	0	0	0	5	5	5
4. Platelet refractoriness, i.e., aplastic anemia, myelodysplastic syndrome (MDS), myelofibrosis, coagulopathic bleeding (n=4)						
-Concordance	2	2	2	1	1	1
-Discrepancy	1	0	1	0	1	0
Total	19	17	18	24	26	25

equal to 0.05 were considered significant. The test strength of agreement was shown by Kappa value (κ). SPSS version 16.0 software was used for statistical analyses.

Results

1. Platelet reactive antibodies in the test samples

Platelet antibodies were tested in the sera of 10 healthy blood donors, considering a negative control group, by LCT, SPRCA and flow cytometry. All sera showed negative platelet reactive antibodies for either HLA or HPA antibodies. In the study samples, 43 patients' sera (32 females and 11 males) were tested with all three techniques. The average age of the subjects was 36.12 years (range 8-75). Twenty-one samples were from haemato-oncology patients who were diagnosed with either leukemia or lymphoma and had several leukocyte contaminating blood component transfusions before developing platelet refractoriness, 13 samples were from idiopathic thrombocytopenic purpura (ITP) patients and five samples were from mothers of babies suffering NAIT. The remaining four samples were from thrombocytopenic patients with underlying aplastic anaemia (1 patient), myelodysplastic syndrome (MDS) (1 patient), myelofibrosis (1 patient) and coagulopathic bleeding (1 patient), who had platelet refractoriness (Table 1).

HLA reactive antibodies were tested with LCT, SPRCA and flow cytometry and HPA reactive antibodies were tested with SPRCA and flow cytometry.

HLA reactive antibodies were found in 18, 17 and 19 out of 43 sera when using LCT, SPRCA and flow cytometry techniques, respectively. There were 16 sera which had similarly positive HLA reactive antibodies tested by the three techniques. Discrepancies in HLA reactive antibodies results were found in four sera (Table 1). Three sera were reactive by flow cytometry and two of these sera were also reactive by LCT. One serum was weakly reactive only when tested by SPRCA and reactivity was found only in 2 out of 6 screened target platelets but negative when tested by flow cytometry and LCT. One serum for which flow cytometry tested reactive against a platelet panel was negative with LCT. The test was repeated using flow cytometry using six mononuclear cells as target cells, to confirm the presence of HLA reactive antibodies in the serum. The results showed positive HLA antibody so this was a false negative reaction by LCT.

HPA reactive antibodies against chloroquine-treated platelets were found in four out of 43 sera when tested by the flow cytometry technique, while there were only two reactive sera with SPRCA. These four sera were also reactive



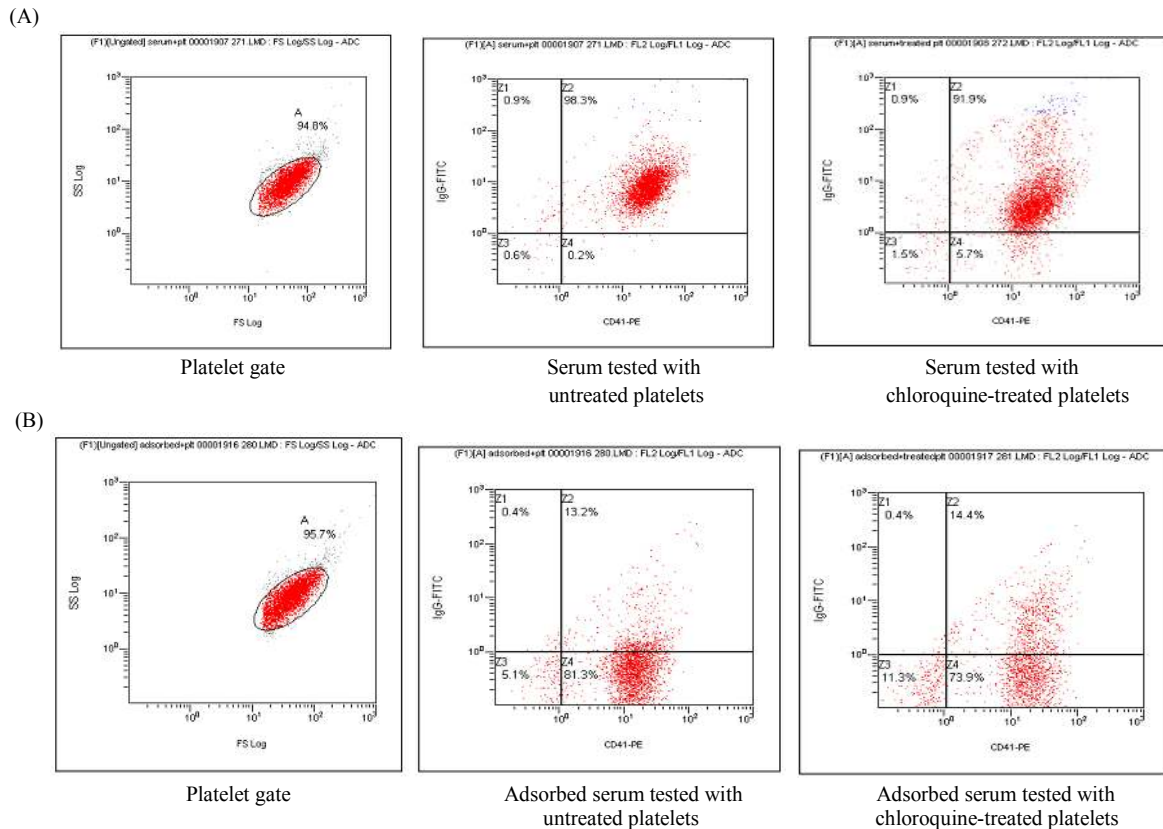


Figure 1. Result of flow cytometry study on the serum of the mother of a NAIT baby which showed positive reactions against both chloroquine-treated and untreated platelets (A). After serum was adsorbed by chloroquine-treated platelets, flow cytometry study was repeated in adsorbed serum and negative reactions against both chloroquine-treated and untreated platelets were observed (B).

against untreated platelets during flow cytometry, but when tested by LCT, they were non-reactive. The combination of HLA and HPA reactive antibodies were studied using flow cytometry. Firstly, the reactive serum was adsorbed by chloroquine-treated platelets and then the adsorbed serum was tested against untreated and chloroquine-treated platelets. The studied serum became non-reactive against both panels of platelets, a result consistent with the single HPA antibody (Figure 1.). Therefore, SPRCA yielded two false negative reactions in these four samples (Table 2).

2. Sensitivity and specificity analyses of the assays

A comparison of the sensitivity and specificity of the three techniques was carried out. In the analyses of sensitivity and specificity of the methods testing for HLA antibodies, flow cytometry was taken as the reference. The sensitivity and specificity were calculated as described in Materials and Methods.

According to the SPRCA vs. flow cytometry comparison, SPRCA confirmed the presence of HLA antibodies in 16 sera and their absence in 23 sera. These were considered true positive and true negative results. One sample was negative with flow cytometry but (false) positive with SPRCA. Additionally, three sera were false negatives with SPRCA, therefore, the sensitivity and specificity of SPRCA were 84.21% and 95.83%, respectively.

When comparing LCT to flow cytometry, it was observed that LCT reported HLA antibody true positives in 18 sera and true negatives in 24 sera. One sample showed a positive reaction by flow cytometry but a negative reaction by LCT (LCT false negative). Consequently, the LCT sensitivity and specificity were 94.73% and 100%, respectively.

In the study of HPA antibodies, the sensitivity and specificity of SPRCA were assessed using the flow cytometry technique as a reference. Two sera showed positive results by both SPRCA and flow



cytometry in chloroquine-treated platelets and two sera did not show HPA antibodies by SPRCA but were HPA antibody positive by flow cytometry. Both techniques confirmed 39 negative sera, thus the sensitivity and specificity of SPRCA were 50% and 100%, respectively.

The LCT and SPRCA testing results in comparison with flow cytometry were statistically analyzed. A 2x2 table was generated and the chi-square test (χ^2) was performed to determine independence. LCT and SPRCA correlated significantly with flow cytometry ($p < 0.001$), having a good strength of agreement ($\kappa = 0.76$ and $\kappa = 0.81$, respectively).

Table 2. Results of HPA antibody screening using flow cytometry technique (Flow) and solid phase red cell adherence assay (SPRCA) in 43 patients.

History	Reactive samples		Non-reactive samples	
	Flow	SPRCA	Flow	SPRCA
1. Haematological malignancy with platelet refractoriness (n=21)	0	0	21	21
2. Idiopathic thrombocytopenic purpura (n=13)	2	1	11	12
3. Mothers of babies suspected NAIT (n=5)	2	1	3	4
4. Platelet refractoriness, i.e., aplastic anemia, MDS, myelofibrosis, coagulopathic bleeding (n=4)	0	0	4	4
Total	4	2	39	41

Discussion

Our study aimed to assess the sensitivity and specificity of LCT, SPRCA and flow cytometry in determining the presence of platelet-reactive auto- and alloantibodies in patients' sera. The data showed that between LCT, SPRCA and flow cytometry, the latter demonstrated a higher sensitivity and specificity than the other techniques, for both HLA and HPA reactive platelet antibodies. In the healthy control group, blood donors who were never transfused or pregnant, platelet-reactive antibodies were not observed. As shown in this study, when ITP patients are not taken into account, HLA alloimmunisation is a major clinical problem in patients with platelet refractoriness (60% of patients). This information is consistent with that from other studies, which have found HLA

alloimmunisation rates at around 30% to 70% in patients with platelet refractoriness^{14,17,18}.

Regarding HLA antibody screening, the flow cytometry technique detected antibodies more frequently than LCT and SPRCA. Considering the flow cytometry technique as a reference, the sensitivity and specificity of SPRCA were 84.21% and 95.83%, respectively and the sensitivity and specificity of LCT were 94.73% and 100%, respectively, with significant correlation between the three methods. These data suggest that flow cytometry is more sensitive than LCT and SPRCA for HLA antibody screening, a finding consistent with those of other studies. Freedman and colleagues compared LCT and SPRCA with the flow cytometry technique in platelet crossmatching and found that the flow cytometry technique had the best sensitivity and specificity¹⁹. Kohler and colleagues reported that sensitivity/specificity of flow cytometry technique were 94.7% and the 96.3% respectively when MAIPA was taken as a reference indicated that sensitivity/specificity of flow cytometry is approaching that by the MAIPA assay¹⁶. This is a reason why we used flow cytometry as the reference method. Although MAIPA was shown to be the best sensitivity/specificity technique for HLA and HPA associated platelet antibody detection, practically it is a complicated procedure to perform as a routine test. Therefore, flow cytometry is considered the most sensitive and specific technique for routine use.

The HPA reactive antibody study, revealed four reactive sera against both chloroquine-treated and untreated platelets. Two sera did not show positive reactions by SPRCA but did so by flow cytometry. These sera were studied to classify the antibodies, as directed against either HPA alone or the combination of HPA plus HLA antibodies. To do this, the serum was adsorbed with chloroquine-treated platelets and then the adsorbed serum was tested with untreated and chloroquine-treated platelets using the flow cytometry technique. The results proved negative in both platelet panels, indicating the serum antibodies were targeting HPA alone (Figure 1.) and further confirming the inferiority of SPRCA sensitivity to that of the flow cytometry technique. The SPRCA sensitivity and specificity for HPA antibody screening were 50% and 100%, respectively and a possible explanation for the low sensitivity of SPRCA is the small sample size

of sera containing anti-HPA positive antibodies, which might affect the sensitivity accuracy of the test. In future studies, the number of samples for anti-HPA antibodies should be large enough to provide adequate authority for conclusions to be reached.

Two sera from patients, whose provisional diagnoses were NAIT and idiopathic thrombocytopenia, were negative for anti-HPA antibody by SPRCA, but reactive with flow cytometry. The finding that SPRCA was not sensitive enough to diagnose NAIT, the risk of which in foetuses/newborn is very high, is crucial, so SPRCA should not be used for NAIT investigation. Similarly in HLA antibody screening in potential organ transplant recipients, a high sensitivity technique such as flow cytometry should be used. However, SPRCA may still have a role where the flow cytometry test, which needs expensive instrumentation, is not available, in providing compatible platelet transfusion in patients who have platelet refractoriness.

Flow cytometry is a novel technique and has been widely used to detect antigens or antibodies on cell surfaces. The principle of this technique is to measure the fluorescence intensity emitted from a laser activated fluorescein dye bound to antibodies in order to determine specific antigens. Flow cytometry is a reliable and objective method compared to other methods such as LCT and SPRCA which need skilled personnel and the analysis using these methods is likely to be more subjective. Furthermore, flow cytometry analyses fresh platelets in which platelet antigens may be intact. This is an advantage in the detection of some platelet reactive alloantibodies specific for labile epitopes which can be detected only on intact platelets. The plausible explanation for the negative SPRCA findings in some samples of our study was the freeze and thaw process in the platelet preparation (lysate platelets) which may have affected the targeting epitopes on the platelet membrane such as the labile component of HPA antigens (HPA-3)²³⁻²⁴ and the small numbers of retained platelet antigen molecules (HPA-5 has 1,000-2,000 sites per platelet)². The LCT study on HLA antibody detection showed that it is less sensitive than flow cytometry. Furthermore, the disadvantages of this technique were that it could not detect the HPA antibody, was time consuming and requiring skilled personnel. We concluded

that flow cytometry had advantages in detecting platelet reactive alloantibodies associated NAIT and post transfusion purpura. For HLA alloantibody screening in a potential transplantation recipient the flow cytometry technique was superior to the LCT when a mononuclear cell panel was used instead of a panel of target platelets because mononuclear cells have stronger HLA expression than the expression on platelets⁹.

The specificity of antibodies in sera with positive platelet antibody screening was not studied because of limited target cells resources.

In conclusion our study found that LCT and SPRCA had a lower sensitivity and specificity in detecting platelet reactive antibodies against both HLA and HPA antibodies than that of flow cytometry. In addition, the flow cytometry technique was quick, required only a small amount of serum and was easy to perform and simple to interpret. In summary, consistent with previous reports, the flow cytometry technique is suitable for routine investigations of alloimmune causes of platelet transfusion refractoriness including NAIT, PTP and HLA antibody screening in a potential organ transplant recipient.

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