

## Original Article

# Comparative of three methods (ELIZA, MAIPA and flow cytometry) to determine anti-platelet antibody in children with ITP

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Received August 20, 2014; Accepted September 15, 2014; Epub December 15, 2014; Published December 30, 2014

**Abstract:** Immune (idiopathic) thrombocytopenic purpura (ITP) is an autoimmune disease characterized by the increased anti-platelet antibodies in the patient's sera and decreased platelets in the blood circulation. This study has determined and characterized the antiplatelet glycoproteins in children with ITP. Thirty eight children, who were hospitalized with clinical signs of ITP in Mofid Children Hospital (Tehran, Iran) during 18 months, went under our clinical studies in a research project. ELISA, Flow cytometry and MAIPA (Monoclonal Antibody Immobilization of Platelet Antigens) methods were employed to determine serum anti-platelet antibodies level. The anti-platelet antibodies level above mean + 3SD of control group was assumed as positive. The platelet counts ranged between  $2 \times 10^9/L$  and  $100 \times 10^9/L$ . Among the patients 63.5% of them were anti-platelet antibodies positive with ELISA method. Results of platelet lysate method showed that 51.7% of patients had antibodies against platelet antigens. Antibody against platelet GPIIb/IIIa, GPIb/IX and GPIa/IIa using MAIPA method were 48%, 54% and 25% respectively. In flow cytometry 62% of patients showed anti-platelet antibodies. The comparison of three methods shows that since MAIPA is the specific method for the detection of very small amount of antibody against glycoprotein antigens, it has the advantage of differentiating between immune and non-immune thrombocytopenia.

**Keywords:** Anti-platelet antibody, ELIZA, MAIPA, flow cytometry

## Introduction

Immune thrombocytopenia purpura (ITP) is an autoimmune disease characterized by loss of self tolerance leading to the production of auto antibodies directed against platelet antigens [1-3]. Acute thrombocytopenia purpura mostly was reported in children under 16 years old. Kuhne and colleagues reported that the mean age of children with ITP at presentation was 5.7 years. Approximately 70% were ages 1 to 10 years with 10% of the cohort infants between 3 and 12 months old and the remainder 20% older children (ages 10 to 16 years) [4]. By now we have a clear understanding that for most patients with ITP, thrombocytopenia results from both increased platelet destruction and decreased platelet production [5]. The auto

antibodies produced against platelet glycoproteins are able to bind to platelet membranes, initiating pathways that result in dysfunction and destruction of platelets and clinical sings. These include petechiae, ecchymosis, and bleeding in some patients [3]. Recently, primary ITP has been uniformly defined as an autoimmune disorder characterized by an isolated platelet count lower than  $100 \times 10^9/L$  [6]. Therefore by this definition we know that the most autoantibodies in patients who have ITP could be identified by the IgG class with specificity against platelets, glycoproteins IIb/IIIa and Ib/IX [7]. Around 85% of platelet auto antigens lie on the platelet GPIIb/IIIa, GPIb/IX or GP Ia/IIa complexes, and 15% of them are found on other membrane glycoproteins [8]. Although, the detection of platelet autoantibodies is difficult

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and not available routinely in most clinical hematology laboratories [7] even with the best direct tests performed in expert laboratories, Anti-platelet antibodies have been found in the sera of 80% of ITP patients [9]. However, despite many studies on antiplatelet antibodies, characterization of binding and evaluation of anti-platelet auto antibodies remain poor [10] and many questions remain unanswered [3]. The aim of our study is to determine and characterized the anti-platelet antibodies in children with ITP with different methods and procedures.

### Materials and methods

#### *Patients*

38 children who were hospitalized with clinical signs of ITP in Mofid children hospital during 18 months, all of them were under study in our project. The physician in clinic recorded the patients' information including age, gender, history and clinical signs such as petechiae, purpura and ecchymosis in a standard format. All the relatives gave us consent to take blood sample from their children.

#### *Samples preparation*

For platelet count, 2 ml anticoagulated [Ethylen Diamine Tetraacetic Acid (EDTA)] blood was collected and counted by a cell counter (Sysmex). To detect anti-platelet glycoproteins antibodies, 3 ml serum was obtained from patients' clotted blood and aliquoted into two tubes and stored in freezer.

#### *Preparation of whole platelets*

Anti-coagulated [Acid Citrate Dextrose (ACD)] blood was collected from healthy O negative blood type volunteers and centrifuged at 200 g for 10 minutes. The platelet-rich plasma (PRP) was removed and recentrifuged at 1200 g for 10 minutes. After four time washing with phosphate buffer saline (PBS) pH 7.4, the sedimented platelets were re-suspended in buffer containing 10% dimethylethyl sulfoxide (DMSO) and aliquoted into a cryotubes and stored in liquid nitrogen.

#### *Preparation of platelet lysate*

Washed platelets were lysed with 6 ml of lysis buffer (20 mmol Tris, 150 mmol NaCl, 1 mmol MgCl<sub>2</sub>, 1 mmol CaCl<sub>2</sub>, pH, 7.4 and 1% Triton X

100) containing anti-protease and incubated at 4°C for one hour. Centrifuged at 15,000 g for 30 minutes at 4°C Supernatant was removed by centrifuging at 4000 for 40 minutes and concentrated with (10,000 MW Cut off) filter. The concentration of platelet membrane proteins were determined using Bradford assay.

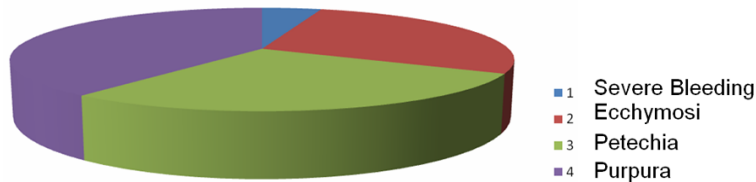
#### *Anti-platelet antibodies detection*

ELISA plate was coated with 50 µL of washed platelet suspension containing  $3 \times 10^8$  platelets in bicarbonate buffer (pH 8.6) in each well or 100 µL of platelet lysate (200 µg/ml) and incubated at 4°C overnight. The following day plate was centrifuged at 2000 g for 5 minutes. After washing and blocking with 3% BSA in PBS and incubating for an hour at room temperature (RT), 100 µL of diluted serum of patients and controls (1:10 diluted in PBS) were added and the plate was incubated for an hr at 37°C. The unbound anti-platelet antibodies were removed by washing four times with PBS 0.05% Tween 20 (PBST washing buffer). 100 µL Goat anti-human HRP-conjugated antibody (1:50 000 diluted in PBS) ((Serotech Co. UK)) was added to each well and incubated at RT for an hour. The plate was washed five times with washing buffer then 100 µL of substrate [Tetramethylbenzidine (TMB) sigma] was added and the plate incubated for 30 min in a dark place. The reaction was stopped by adding 100 µL of 3 M HCL, and the OD was measured at 650 nm.

#### *MAIPA (monoclonal antibody immobilization of platelet antigens)*

ELISA plate was coated with 100 µL of Goat anti mouse IgG [final concentration 5 µg/ml diluted in Carbonate Bicarbonate Buffer (CBB) pH 8.6] in each well and incubated overnight at 4°C. After washing with Tris Buffer Saline (TBS), the plate was blocked with 200 µL of 3% BSA in PBS and incubated for an hr at room temperature (RT). 100 µL of washed O negative platelets suspension containing  $5 \times 10^{11}$ /L were added to the tubes which were blocked with 50 µL 3% BSA and incubated for an hour with 100 µL of patients and controls serum for an hour at 37°C. 40 µL of each monoclonal mouse anti platelet-glycoproteins [IIb/IIIa, Ib/IX and IIa/IIa (Novacastra UK)] with final concentration of 20 µg/ml diluted in PBS were added to the individual tubes containing mixture of washed platelets and patients or controls sera and incubated at 37°C for 30 minutes.

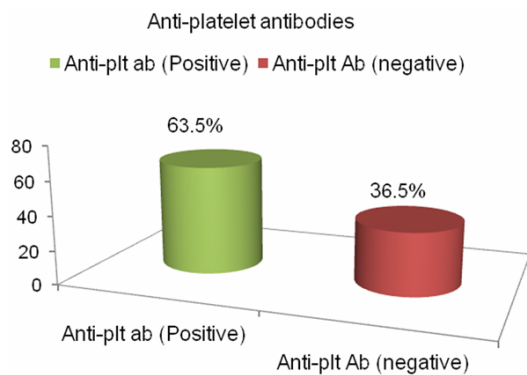
## Anti-platelet antibody in children with ITP



**Figure 1.** A Pie Chart of the ITP Patients' Clinical signs

**Table 1.** The platelet count in patients

Platelets count	NO
$95 \times 10^9/L$ -51	7
$31-50 \times 10^9/L$	5
$10-30 \times 10^9/L$	18
$< 10 \times 10^9/L$	8



**Figure 2.** ITP Patients who were Anti-platelet antibodies positive against whole platelet, Anti-platelet antibodies against platelet antigens of 63.5% of patients were detected (green color).

The unbound human anti platelet-glycoproteins were removed by washing three times with TBS 0.05% Tween 20 (TBST washing buffer). 100  $\mu$ L of lysis buffer (TBS containing 0.05% Nonidet P-40 Sigma) were added to each tubes and incubated for 30 minutes at 37°C.

To prevent protein destruction, anti-protease agents was added to lysis buffer. After centrifuging all tubes at 14 000 g for 30 minutes at 4°C, 100  $\mu$ L of supernatant was added to each well which were coated with anti-mouse antibody and incubated at 4°C for 90 minutes. The plate was washed four times with washing buffer and 100  $\mu$ L of Goat antihuman HRP-conjugated antibody (1:50 000 diluted in PBS) (Serotech Co. UK) was added to each well and incubated at RT for an hour. The plate was washed five times with washing buffer. 100  $\mu$ L

of substrate (TMB) was then added and the plate incubated for 30 min in a dark place. The reaction was stopped by adding 100  $\mu$ L of 3 M HCL, and the OD (optical density) was measured at 650 nm.

### Flow cytometry

The platelets of a O<sup>-</sup> (Rh negative) volunteer were prepared and washed as described above. The final concentration of platelets was  $5 \times 10^{11}/L$ . 100  $\mu$ L of washed platelets were incubated for 1 h at 37°C with 100  $\mu$ L of patients and controls serum in the tubes. The platelets were washed four times with Tyrode's buffer by centrifuging for 5 min at 1200 g. Bounded anti-platelet antibodies was detected with 100  $\mu$ L of dilute antihuman FITC-conjugated antibody (1:40 diluted in PBS) (Zymed Co. USA) which was added to the samples and the negative control tube. Five microlitre of anti-6GPIIb/IIIa FITC-conjugated antibody (Serotech Co. UK) was added to the positive control tube and all tubes were incubated for 30 min at room temperature in the dark condition. After washing five times with Tyrode's buffer, 1 milliliter of paraformaldehyed was added to each tubes and then analyzed by7FACScan (Partec) using the following thresholds: Forward Scatter Side (FSC): E01, Side Scatter (SSC): 350 and Fluorescence (FL1): 400.

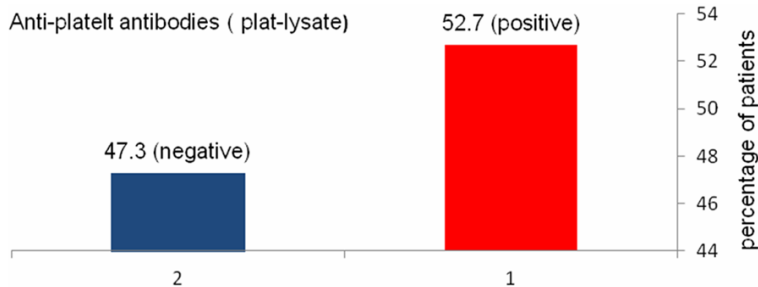
### Statistical analysis

The SPSS software, version 16.0, was used for data analysis. For descriptive purposes, we used mean (SD) for quantitative variables and No (%) for qualitative variables. In addition, we utilized the non-parametric Spearman's correlation coefficient to show the relationship between two variables under study (for instance, antibody level and clinical characterizations).

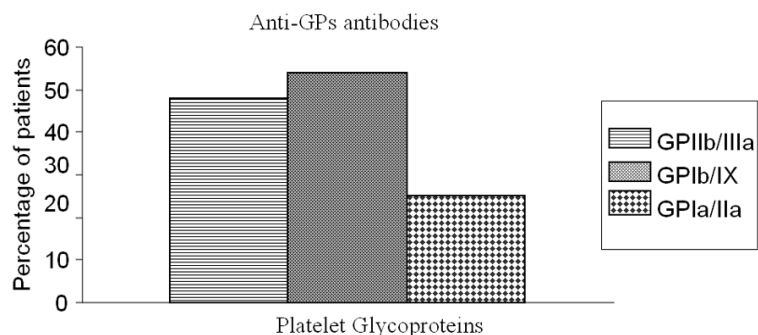
### Results

In this project, the goal of our study is to determine anti-platelet antibodies in children with ITP. In the study, 38 children who were hospitalized in Mofid Children's Hospital with ITP clinical signs during 18 months and 12 healthy people as a negative control group were chosen. Sixty eight percent of patients were girls and 32% boys and their age ranged from less than a year, up to sixteen years old. The clinical

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**Figure 3.** ITP Patients who were Anti-platelet antibodies positive against platelet lysate, Anti-platelet antibodies against platelet antigens of 52.7% of patients were detected (red color).



**Figure 4.** ITP Patients who were shown antibodies positive against platelet glycoproteins.

signs were variable among the children; 10 individuals only with petechiae symptom, 24 of them showed purpura and ecchymosis signs in addition to petechiae, and finally 4 of those children, were found to have severe bleeding from gums and urethral tract. **Figure 1**, shows more details about clinical signs in ITP patients.

Peripheral platelet count, ranges from less than  $3 \times 10^9/L$  up to  $95 \times 10^9/L$ . **Table 1**, shows more details about platelet count in patients.

The results with ELISA, indicated that anti-platelet antibodies in sera of all patients either with clinical signs and low platelets counts or just low platelets counts reacted strongly with whole platelet antigens. It should be mentioned that we used peroxidase activity to calculate level of antibody instead of OD (optical density) by following formula:

$$\text{Percent change} = \frac{\text{OD sample} - \text{OD control}}{\text{OD control}} \times 100$$

The mean controls absorbance was used as normal value. We also used the mean controls antibody level with 3 standard deviation (mean

+ 3SD) as the cut off. The anti-platelet antibodies level of each sample above the cut off was assumed as antibody positive.

Among 38 patients, 63.5% had anti-platelet antibody positive with mean platelet count of  $41.5 \times 10^9/L$  using non parametric spearman coefficient, it was statistically shown that there was a significant correlation between clinical signs and antibodies level in patients ( $r = 0.45$ ,  $P < 0.05$ ). **Figure 2**, shows the anti-platelet antibodies in ITP patients.

Results of platelet lysate method showed that 52.7% of patients had antibodies against platelet antigens which are in a good correlation ( $r = 0.38$ ;  $P < 0.04$ ) with the severity of clinical signs. **Figure 3**, shows the anti-platelet antibodies against platelet lysate in ITP patients.

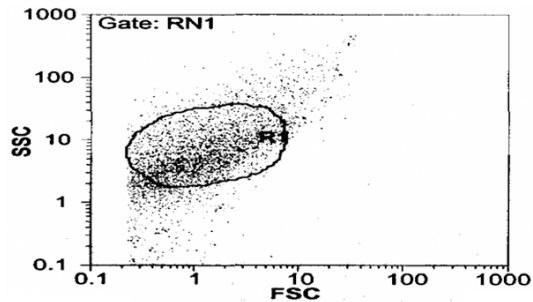
### The determination of anti-platelet GP antibodies

Using MAIPA method, antibodies in patients' sera were analyzed against three most important platelet glycoproteins (GP IIb/IIIa, IIa/Ia, Ib/IX). Results have shown that 48% of patients were anti-GP IIb/IIIa antibody positive with mean platelet count of  $38 \times 10^9/L$ . 54% were anti-GP Ib/IX positive with mean platelet count of  $50 \times 10^9/L$ . The correlation between anti-GP Ib/IX positive and clinical signs was 0.2. Out of 38 patients only 25% were anti GP-Ia/IIa positive with mean platelet count of  $48 \times 10^9/L$ . Using non parametric spearman coefficient, it was statistically shown that a significant correlation was seen between increased anti-GP Ia/IIa level and the platelet count in patients ( $r = 0.35$ ,  $P < 0.05$ ). **Figure 4**, shows the anti-Gps antibodies in ITP patients.

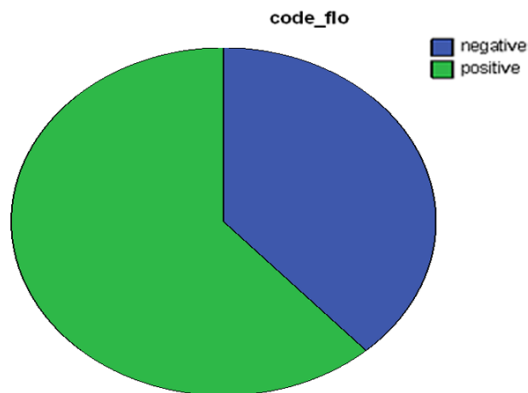
### Flow cytometric detection of anti-platelets antibodies

In order to determine anti-platelet antibodies, we employed flow cytometry.

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**Figure 5.** Platelet gating using Fluorescent anti-GPII-Ia has shown SSC Versace FSC.



**Figure 6.** Chart for ITP Patients who were shown 62% Fluorescence antibodies positive.

Anti-GP IIIa was used to determinate the platelet gating and as a positive control. The result showed that 94% of washed platelets were in the platelet gate the **Figure 5**, demonstrate platelet gating. We also used 12 healthy adult volunteered sera as negative anti-platelet antibodies. The mean fluorescence intensity (MFI) of negative controls with 3 standard deviation (+ 3SD) used as a flow cytometric cut off. The MFI of positive control was 98%. 62% of patients were anti-platelet antibodies positive by flow cytometry. **Figure 6**, shows the anti-platelet antibodies in ITP patients.

### Discussion

ITP is an autoimmune disease characterized by platelet destruction, which can result in increased bleeding and haemorrhage [2, 3, 10]. For further characterization function of auto antibodies against platelet glycoproteins in ITP patients, our studies have indicated, first of all the determination of anti-platelet antibodies using ELIZA method, then the detection of

these antibodies by flow cytometry and compares the result with ELIZA assay and finally the detection of anti-GPs antibodies by MAIPA.

During 18 months period, 38 children with ITP clinical symptoms were studied. The result of our clinical studies indicated that major signs (34 Children) were petecheaia and purpura with minor signs of (4 Children) severe bleeding. The age variety was between 1 to 16 years, which 68% of them were under 8 years old. The platelet count showed that more than 50% of patients had platelet count  $< 30 \times 10^9/L$ , which this factor makes severity in clinical signs. Results Data from 225 children in UK were analyzed at 6 months follow-up, 32% had a persistent platelet count  $< 150 \times 10^9/L$ , but only 4.8% had a count  $< 20 \times 10^9/L$  [10]. In some patients, not only their platelets have been decreased but also they had anemia due to the decrease of the RBC and hemoglobin. The normochrmic normocytic anemia in these patients might be due to losing blood as result of hemorrhage and massive ecchymose or probably they had ITP with autoimmune hemolytic anemia (Evans syndrome).

Twelve normal voluntary adults were used as the normal controls and the main result of anti-platelet antibodies + 3SD (Mean + 3SD) was used as the cut off, and ITP patients who their anti-platelet antibodies were above the cut off line placed with patients with antibody positive. It was found that 63.5% out of 38 patients in our assay were anti-platelet antibodies positive with the main platelets count  $41.5 \times 10^9/L$  which show a good correlation between decreasing circulated platelets and increasing antibodies, compare with anti-platelet negative patients. There was a positive correlation between increasing serum anti-platelet antibodies and clinical sings. In some cases there were not relations between anti-platelet antibodies and platelet account or clinical signs. This could be due to the acute phase of disease which their platelets absorbs antibodies and removes these platelet sensitivities from circulation by phagocyte system. In a large study, less than 7% of thrombocytopenic pregnant women were found to have auto-antibodies. Thus, the most useful means of differentiating between these syndromes are by definition, the antenatal history [11, 12]. In the other hand, detection of auto antibodies on platelet surface

due to platelet structure is not simple [13]. However, using the platelet lysate as source of platelet antigens, 52.7% of patients were antibodies positive. In comparison with the whole platelet as a source of antigens, 11% of them failed to react with antibodies. It is suggested that in the platelet lysate perhaps, the conformational structure of platelet glycoproteins was changed, resulting in not expression of more platelet antigens [14, 15]. The result showed that 62% of patients were anti-platelet antibodies positive by flow cytometry. The correlation of anti-platelet antibodies positive in patients by ELISA and Flow cytometry assays was 0.3. Although detection of anti-platelet antibodies by flow cytometry is more available than ELISA [16-19] but the sensitivity of ELISA is high. However, it would be suggested that using of two techniques are more assuring. We employed MAIPA technique to detect anti-platelet glycoproteins. The results showed that 48% of patients were anti-GPIIb/IIIa positive. The correlation between this antibody and antibodies against whole platelets was 0.4. Van Leuven has reported that 80% out of 42, ITP patients were anti-GPIIb/IIIa positive [20].

In our study 54% of patients were anti-GPIIb/IX positive. It needs to be mentioned that previously have been reported that 35% patients, had both anti-GPIIb/IIIa and Ib/IX antibodies in their sera [21]. Fuji Sawa and colleagues have found that in 75% of ITP patients' anti-GP IIb/IIIa and Ib/IX [22]. In 25% of patients we have found anti-GPIa/IIa and correlation between level of sera anti-GPIa/IIa and low platelet count was 0.4. Ho WL et al, found that patients without antecedent of preceding infection (API) were more likely to have anti-GPIa/IIa than those with API (42.9% vs. 5.5%, (P = 0.048) [20]. However the determination of antibodies against the platelet glycoproteins by MAIPA is very sensitive though it may take a long time [23-27]. Finally, the pathogenesis and diagnoses of ITP has proven complex and concurs with several issues such as: genetic predisposition, underlying autoimmune repertoire, inhibition of platelet production, perturbations of cell mediated effector and effector pathways, sequestered harbors within lymphoid organs, and responsiveness to intervention [28].

### Conclusions

- Coating platelets at the bottom of ELISA plate is difficult; we have modified the method by

centrifuging the plate and fixing them with Glutaraldehyde.

- Instead of using optical density (OD) for analysis of the data which can not differentiate low absorption, we used percentage of peroxidase activity.

- The preference of MAIPA method is due to the large amount of Ag-Ab complex in the supernatant of platelet lysate (the second process) and the detection of very small amount of antibody.

- Since MAIPA is the specific method for the detection of antibody against glycoprotein antigens, it has the advantage of differentiating between immune and non-immune thrombocytopenia.

### Acknowledgements

The authors would like to thank the Deputy of research of Shahid Beheshti University of Medical Sciences and Mofid Children's Hospital for kindly supported this research.

### Disclosure of conflict of interest

The authors declare that they have no conflicting interests. Contribution: MH, GK, NT, BBS and RH designed the research, analyzed the data, performed the research and wrote the paper, review and final approval of the version to be published.

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