



FLAER Based Assay According to Newer Guidelines Increases Sensitivity of PNH Clone Detection

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Abstract Flow cytometry has become ‘gold standard’ for detecting abnormal clones in paroxysmal nocturnal hemoglobinuria (PNH), aplastic anemia (AA) and myelodysplastic syndrome (MDS). This pilot study was conducted in 2015 with a primary aim to evaluate the utility of single tube fluorescent aerolysin (FLAER) based testing and its comparison with two tubes non-FLAER based testing (CD55, CD59, CD24 and CD66b) in detecting abnormal PNH clones in these newly diagnosed cases. The secondary aim was an attempt to distinguish PNH from AA/MDS cases associated with PNH clones based on clinical, laboratory features and clone size at diagnosis. In this study, the abnormal PNH clones were detected using a single tube FLAER based testing and two tubes non-FLAER based testing in all cases of PNH ($n = 12$), healthy subjects ($n = 18$) and AA/MDS with PNH clone ($n = 9$) and compared with clinical and laboratory features at diagnosis. The receiver operator curve (ROC) analysis defined the optimal cut-offs for FLAER in granulocytes ($> 0.7\%$) and monocytes ($> 0.9\%$). There was significant positive correlation between FLAER and non-FLAER based testing in these cells ($r > 0.3$ and $p < 0.05$). FLAER based testing helped us in picking up smaller clones which were missed by latter technique in four patients thereby increasing its sensitivity and also technically proved to be cost-effective (Rs. 1800 vs. Rs. 2100). Even in PNH patients, the clone size was slightly higher by using FLAER when compared to non-FLAER based antibodies

panel. The clone size of monocytes was always higher than granulocytes in both PNH and AA/MDS groups. Bone marrow cellularity and mean size of granulocytes and monocytes clone at diagnosis showed a striking statistically significant ‘ p ’ value of < 0.0001 between these groups. In this pilot study, a single tube FLAER based PNH testing had improved clone detection in all cases of PNH, AA/MDS with PNH clones. The clone size was $> 30\%$ in majority of PNH cases whereas in AA/MDS, it was usually $< 10\%$ at diagnosis. Hence this newer technique not only increased the sensitivity of PNH clone detection but also proved to be cost-effective.

Keywords FLAER · Paroxysmal nocturnal hemoglobinuria · Aplastic anemia · Myelodysplastic syndrome

Introduction

Paroxysmal nocturnal hemoglobinuria (PNH) is a rare, acquired clonal hematopoietic stem cell disease with a mutation in a gene called phosphatidylinositol glycan anchor biosynthesis, class A (*PIG-A*). This leads to partial or absolute deficiency of all glycoposphatidyl-inositol-anchor proteins (GPI-AP) [1]. Clinically, this disease manifests as bone marrow failure, hemolytic anemia, smooth muscle dystonia and thrombosis [2].

The laboratory work-up for diagnosing PNH are usually carried out by various tests such as modified Ham’s test, sucrose lysis test, gel card technique (GCT) and flow cytometric methods [3]. Flow cytometry has become ‘gold standard’ for making PNH diagnosis. Numerous monoclonal antibodies are available for flowcytometry such as CD55, CD59, CD24 and CD66b; however the utility of

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these markers seems to be decreasing. Recently, bacterial aerolysin tagged with fluorescent antibody (FLAER) is increasingly used which binds specifically to the GPI moiety of cell surface GPI-linked molecules and causes lysis of normal but not GPI-deficient PNH cells [3–5]. In this study, our primary aim was to study the utility of single tube FLAER based test on granulocytes and monocytes by flow cytometry in all newly diagnosed cases with PNH clone and to compare their results with the our existing two tubes non-FLAER based testing comprised of antibodies such as CD55 (decay accelerating factor, DAF), CD59 (membrane inhibitor of reactive lysis, MIRL), CD24 and CD66b.

The importance of identifying the PNH clones in different clinical context helps in appropriate classification and this is usually done according to guidelines given by International PNH interest group (IPIG) [6]. They are (1) Classical PNH is characterised by the clinical evidence of intravascular hemolysis, presence of large granulocyte clone and the majority of the RBCs are of type III (complete GPI-AP deficiency) PNH cells; (2) PNH arising in the setting of other bone marrow disorders such as aplastic anemia (AA) and myelodysplastic syndrome (MDS); (3) Subclinical PNH (PNH-sc), highly sensitive flow cytometer is required to diagnose this entity because of the presence of smaller clone size along with other bone marrow disorders. However overt symptoms of PNH are not present. This differentiation is also important for using appropriate treatment protocol, particularly in eculizumab era. However this classification scheme had not defined any flow cytometric cut-off for classifying the patients as PNH or other marrow diseases like AA/MDS associated with PNH clones. This prompted us to take up this pilot project with the secondary objective to analyse whether differentiation between PNH and other disorders like AA/MDS with PNH clone was possible or not, based on clinical and laboratory features including PNH clone size at diagnosis.

Materials and Methods

This was a prospective observation study done as a pilot project from June 2014 to December 2015. Totally 39 samples were analysed including twelve newly diagnosed classical PNH cases included as group 1. Nine newly diagnosed cases of AA/MDS (six cases of AA and three cases of MDS) with PNH clones were also included (Group 2). Eighteen age and sex matched healthy subjects were selected as the normal control group (Group 3). This study was approved by Institute Ethics Committee, and is in accordance with the current version of the Helsinki Declaration.

Diagnosis of PNH, AA and MDS

The diagnosis of PNH was made when granulocytes and monocytes had deficient GPI-AP in combination with clinical signs and symptoms. These patients did not have features suggestive of AA/MDS (group 1). We followed the recommendation of International Agranulocytosis and Aplastic Anemia Study (IAAAS) [7] definition for making the diagnosis of AA. Based on the peripheral blood and bone marrow aspiration and biopsy findings, the diagnosis of AA was defined as pancytopenia (Hb < 100 g/L, platelet count < $50 \times 10^9/L$ and ANC < $1.5 \times 10^9/L$) with a hypocellular bone marrow in the absence of an abnormal infiltrate and with no increase in reticulins. The myelodysplastic syndrome (MDS) diagnosis was made according to the World Health Organisation (WHO) 2008 definition on hematopoietic tumors [8]. It is characterised by cytopenia(s), dysplasia in one or more of the major myeloid lineages and ineffective hematopoiesis. If PNH clones were demonstrated in patients with AA or MDS were defined as AA/MDS with PNH clones (group 2).

Laboratory Investigations

Complete Blood Count, Peripheral Smear and Reticulocyte Count

From all the patients and healthy controls, 2 mL of peripheral blood was taken in EDTA tube. The complete blood count (CBC) was obtained from Sysmex-XT-1800i from all the three groups. The peripheral smear was stained with Jenner-Giemsa stain and reticulocyte staining was done with 1% new methylene blue. Absolute reticulocyte count (ARC) was calculated.

Flow Cytometric Detection of PNH Clones

Two-millilitre EDTA-anticoagulated peripheral venous blood sample was collected from all cases of AA, MDS, PNH and healthy controls. The processing and acquisition of sample was done within 4 h of sampling.

Stain-Lyse-Wash-No Fix and Two Tubes Technique by CD55, CD59, CD66b and CD24 [4]

Fifty microlitre of whole blood was delivered into two BD falcon tubes used for performing test. Serial dilutions of the antibodies were used and the final optimal concentrations were obtained from the signal to noise ratio estimation. Five microlitre of CD45-Per CP-Cy 5.5, CD55-FITC (5 μ L) and CD59-PE (5 μ L) were added in one tube (tube 1) and in another tube (tube 2), CD45-Per CP-Cy 5.5 (5 μ L), CD24-PE (7 μ L) and CD66b-FITC (7 μ L) [BD

Pharmingen™] were added and incubated in the dark at room temperature for 30 min. The same volume of blood (50 µL) was taken in third tube without antibodies which was labelled as negative control. Two millilitre of RBC lysing solution was added and kept at room temperature for 10 min. The tubes were centrifuged at 1500 rpm for 5 min, and the supernatant was discarded. The cell button was resuspended in 2 mL sheath fluid, and again centrifuged at 1500 rpm for 5 min. This was followed by thorough washing in sheath fluid and cells were resuspended in 500 µL of sheath fluid.

Lyse-wash-stain-no fix and single tube technique for FLAER based PNH testing [9]

Fifty microlitre of whole blood was delivered into single BD falcon tube for performing the test. Two millilitre of RBC lysing solution was added and kept at room temperature for 10 min. The tube was centrifuged at 1500 rpm for 5 min, and the supernatant was discarded. The cell button was resuspended in 2 mL sheath fluid, and again centrifuged at 1500 rpm for 5 min. Five microlitre of CD45-Per CP cy5.5, CD15-PE Cy7 (3 µL), CD33-APC (3 µL), CD14-APC Cy7 (3 µL), CD24-PE (5 µL) [BD Pharmingen™] and FLAER-FITC (5 µL) [Alexa Fluor® 488 proaerolysin (Cedarlane, USA)] were added and incubated in the dark at room temperature for 30 min. This was followed by thorough washing in sheath fluid and cells were resuspended in 500 µL of sheath fluid. The same volume of blood (50 µL) was taken in other tube, processed similarly but without antibodies and was labelled as negative control.

Acquisition

The cells were acquired on BD FACS Canto II™ (six colour and dual laser). The instrument set-up including PMT voltages, threshold settings, compensation and quality control measures were done using standard methods as recommended by the manufacturer. A minimum of ten thousand events were acquired in each tube for routine clinical laboratory testing. For samples with extremely low leucocyte count, the cells were acquired till the exhaustion of cell suspension.

Analysis

The data was analyzed on BD FACS Diva™ software. In the two tubes technique, double negative CD55 and CD59 population (%) on both neutrophils and monocytes in the first tube; double negative CD24 and CD66b population (%) on only neutrophils in the second tube with already established 1% cut-off was considered positive for PNH clone. In the FLAER based single tube testing, neutrophil

and monocyte gating were done using CD15/SSC and CD33/SSC plot respectively. Double negative FLAER and CD24 population (%) on neutrophils, double negative FLAER and CD14 population (%) on monocytes was considered positive for PNH clone (Fig. 1) and appropriate cut-off was determined in comparison with testing healthy controls.

Bone marrow aspiration (BMA) and biopsy (BMBx)

For all AA/MDS cases, BMA and BMBx were done to assess for overall cellularity and dyspoietic features. Stress cytogenetics was done to rule out inherited bone marrow failure syndromes and conventional cytogenetics in three MDS cases to look for cytogenetic abnormalities.

Statistical Analysis

The data was presented as mean ± standard deviation. Student 't' test was used to compare the data of the different groups. Receiver operator curves (ROC) were used to define cut-off of FLAER based test. Pearson's correlation analysis (for parametric and normally distributed data) or Spearman's correlation analysis (for nonparametric or non-normally distributed data) were used to define the correlation between PNH clone size on granulocytes and monocytes with different hematological parameters. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated for FLAER based technique on both granulocytes and monocytes. Absolute correlation coefficient values (r) of > 0.3 and 'p' values of < 0.05 were taken to indicate statistical significance. Statistical analyses were performed using the MEDCALC software (<http://www.medcalc.be>).

Results

- (i) Cut-off definitions for the PNH clone detection by single tube FLAER based testing:

Using the healthy subjects as disease control, optimal cut-off for the PNH clone detection by FLAER method was obtained from the ROC curve analysis (Fig. 2). The granulocytes clone size of > 0.7% was considered positive with sensitivity and specificity of 95.2% and 100% respectively. The sensitivity and specificity of 90.5% and 100% was obtained when > 0.9% clone size was considered positive for monocytes. In our laboratory, 1% cut-off was already established and considered positive for PNH testing by CD55, CD59, CD66b and CD24.

- (ii) Correlation between single tube FLAER based method and two tubes non-FLAER based method for detecting abnormal PNH clones:

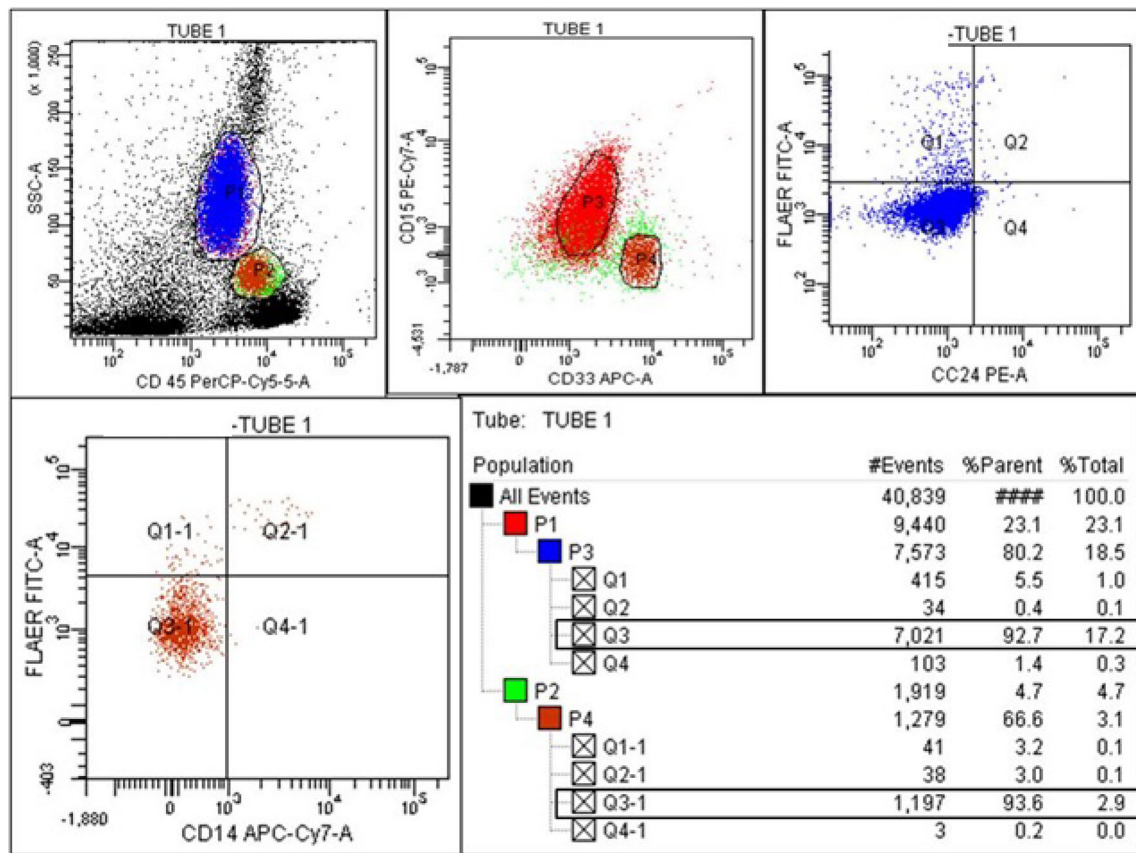
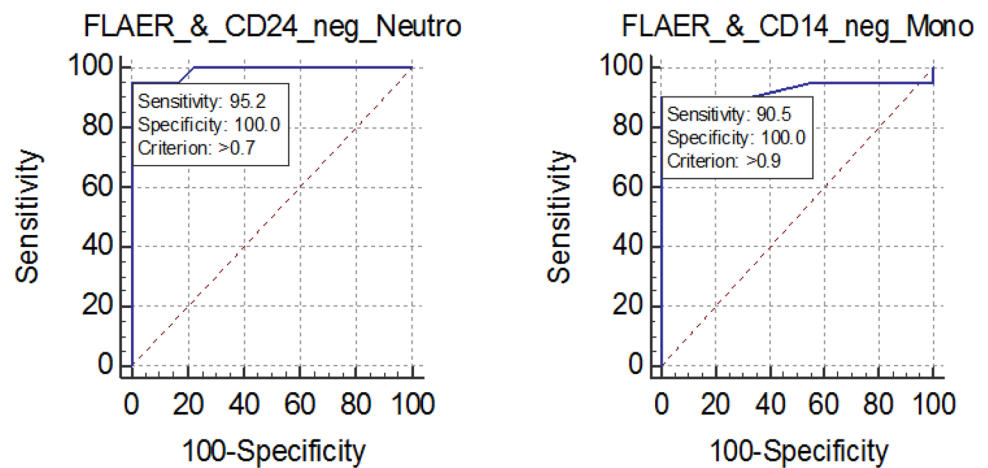


Fig. 1 Scatter plots of PNH case by FLAER based test. In PNH case, CD24-FLAER-deficient granulocytes (92.7%) and CD14-FLAER-deficient monocytes (93.6%) were observed

Fig. 2 Receiver operator curve (ROC) analysis for FLAER based PNH detection



(a) PNH group:

There was a linear correlation between PNH clone size on granulocytes which varied from 14.8 to 84.2% at diagnosis with the clone size on monocytes which varied from 24.2 to 92.7% by FLAER method ($r = 0.8986$; $p = 0.001$). There was a significant positive correlation between single tube FLAER based testing as well as by two tubes non-FLAER based testing such as CD55, CD59,

CD24 and CD66b in both granulocytes ($r = 0.9500$; $p < 0.001$) and monocytes ($r = 0.7839$; $p = 0.0025$).

(b) AA/MDS group with PNH clone:

There was a linear correlation between PNH clone size on granulocytes which varied from 0.8 to 13.9% at diagnosis with the clone size on monocytes which varied from 1.0 to 16.3% by FLAER method ($r = 0.9272$; $p = 0.0003$).

There was a significant positive correlation between single tube FLAER based testing as well as by two tubes non-FLAER based testing such as CD55, CD59, CD24 and CD66b in both granulocytes ($r = 0.8607$; $p = 0.0029$) and monocytes ($r = 0.8032$; $p = 0.0091$). The FLAER method helped us in picking up smaller clone in four patients with AA/MDS which were missed by CD55, CD59, CD24 and CD66b panel of antibodies.

- (iii) Comparison of test efficiencies between single tube FLAER method and two tubes non-FLAER based PNH testing:

In all the patients of PNH cases and AA/MDS cases, FLAER based PNH testing helped us in picking up slightly higher clone size on both granulocytes and monocytes. Using ROC defined cut-off of $> 0.7\%$ and $> 0.9\%$ for granulocytes and monocytes respectively for FLAER method resulted in picking up smaller clones in AA/MDS patients which were otherwise missed by CD55, CD59, CD24 and CD66b testing panel in four patients. This had yielded the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) on granulocytes by CD55, CD59, CD24 and CD66b testing panel were 100%, 44.4%, 70.5% and 100% respectively and on monocytes, these values were 100%, 11.1%, 60% and 100% respectively in the PNH group. The sensitivity was only 55.5% on granulocytes and 88.8% on monocytes in AA/MDS group (Tables 1, 2).

- (iv) Comparison of technical resources utility and cost-benefit analysis:

The lyse-wash-stain-no fix and single tube FLAER technique helped us in reducing the volume of different

antibodies used ranging between 3 μL to 5 μL per test, in contrast to 5 μL to 7 μL per test of various antibodies using stain-lyse-wash-no fix and two tubes technique in the panel of antibodies (tube 1—CD45, CD55, CD59; tube 2—CD45, CD24 and CD66b). Therefore FLAER technique helped us substantially in reducing the reagents and the cost (Rs. 1800/- vs. Rs. 2100/-).

- (v) Clinical and laboratory features of patients with PNH and AA/MDS with PNH clones:

Tables 3 and 4 highlights the various clinical and laboratory features of patients with PNH, AA/MDS with PNH clones and their comparison. Among the clinical features at diagnosis, none of the features showed a statistically significant difference in presentation between PNH group and AA/MDS with PNH clone group. However, bone marrow cellularity and mean size of granulocytes and monocytes clone at diagnosis showed a striking statistically significant 'p' value of < 0.0001 between these groups.

- (vi) Comparisons of PNH clone size by FLAER method in PNH cases and AA/MDS cases:

Paroxysmal nocturnal haemoglobinuria clone size on monocytes was always greater than granulocytes in both the groups. Mean clone size in PNH cases (mean granulocytes = 55.69%, monocytes = 67.51%) was significantly greater than in AA/MDS associated with PNH clones. (Mean granulocytes = 3.46%, monocytes = 5.64%) (Fig. 3). Clone size in PNH patients was always $> 10\%$, and in 83.3% of patients clone size was $> 30\%$ on granulocytes and monocytes. Only two patients (16.6%) had a clone size between 10% and 30% at diagnosis (Table 5). Clone size in AA/MDS associated with PNH clones was

Table 1 The PNH clone detection on granulocytes between FLAER method and CD55, CD59, CD24 and CD66b method

PNH clone detection by routine panel (CD55, CD59, CD24 and CD66b)	Gold standard (FLAER based test) $> 0.7\%$ for granulocytes (positive)		Total
	PNH group	AA/MDS group	
Positive ($> 1\%$ granulocytes)	12	5	17
Negative ($< 1\%$ granulocytes)	0	4	4
Total	12	9	21

Table 2 The PNH clone detection on monocytes between FLAER method and CD55, CD59, CD24 and CD66b method

PNH clone detection by routine panel (CD55, CD59, CD24 and CD66b)	Gold standard (FLAER based test) $> 0.9\%$ for monocytes (positive)		Total
	PNH group	AA/MDS group	
Positive ($> 1\%$ monocytes)	12	8	20
Negative ($< 1\%$ monocytes)	0	1	1
Total	12	9	21

Table 3 Comparison of clinical parameters of PNH patients and AA/MDS with PNH clone

Parameters	PNH (n = 12)	AA/MDS (n = 9)	'p' value
Median age (range) (years)	32.5 (21–52)	42 (9–55)	–
M:F	2:1	8:1	–
<i>Clinical features</i>			
Hemoglobinuria (n, %)	2 (16.6%)	0 (0%)	0.485
Thrombosis (n, %)	4 (33.3%)	0 (0%)	0.103
Abdominal pain (n, %)	3 (25%)	0 (0%)	0.228
Fatigue (n, %)	12 (100%)	9 (100%)	1.000
Fever (n, %)	7 (58.3%)	5 (55.5%)	1.000
Jaundice (n, %)	2 (16.6%)	2 (22.2%)	1.000
Bleeding manifestations (n, %)	9 (75%)	6 (66.6%)	1.000
Transfusion (n, %)	12 (100%)	9 (100%)	1.000
Dysphagia (n, %)	1 (8.3%)	0 (0%)	1.000
Cytopenias (n, %)	12 (100%)	9 (100%)	1.000

Table 4 Comparison of laboratory parameters of PNH patients and AA/MDS with PNH clone

Parameters	PNH (n = 12) (Mean ± SD, range)	AA/MDS (n = 9) (Mean ± SD, range)	'p' value
Hb, g/L	63.2 ± 19.3 (38–98)	73 ± 25.3 (23–112)	0.3403
Total WBC count, ×10 ⁹ /L	3.4 ± 1.8 (1.1–8.8)	3.1 ± 1.6 (0.8–5.8)	0.7382
ANC, ×10 ⁹ /L	1.6 ± 1.5 (0.5–6.1)	1.1 ± 0.8 (0.1–2.5)	0.3822
Platelet count, ×10 ⁹ /L	88 ± 139.02 (10–483)	53.66 ± 91.67 (3–292)	0.5287
Reticulocyte count, %	1.83 ± 2.67 (0.5–10.0)	0.61 ± 0.22 (0.5–1.0)	0.1902
Absolute reticulocyte count, ×10 ⁹ /L	38.54 ± 17.83 (4.21–67.39)	6.81 ± 2.68 (1.70–11.19)	0.1790
<i>Bone marrow cellularity</i>			
Normocellular for age (> 40%)	8	0	0.0046
Hypocellular for age (20–40%)	2	3	0.6107
Hypocellular for age (< 20%)	2	6	0.0318
<i>Mean ± SD of PNH clone at diagnosis (%)</i>			
Granulocytes	55.69 ± 22.76 (14.8–84.2)	3.46 ± 4.26 (0.8–13.9)	< 0.0001
Monocytes	67.51 ± 23.52 (24.2–92.7)	5.64 ± 5.19 (1.0–16.3)	< 0.0001

usually < 10% at diagnosis. Only one patient (11.1%) had a clone size between 10% and 30% on granulocytes and two patients (22.2%) had a clone size between 10% and 30% on monocytes at diagnosis. None of the patients had a clone size > 30% at diagnosis in this subgroup (Table 5).

Discussion

The primary aim was to study the utility of single tube FLAER based test and to compare their results with the already existing two tubes non-FLAER based testing. Ours being a routine clinical laboratory, already established cut-off of 1% was used for two tube non-FLAER based technique. The ROC curve helped us in establishing the newer cut-off of 0.7% and 0.9% as positive for granulocytes and monocytes respectively in the FLAER based test.

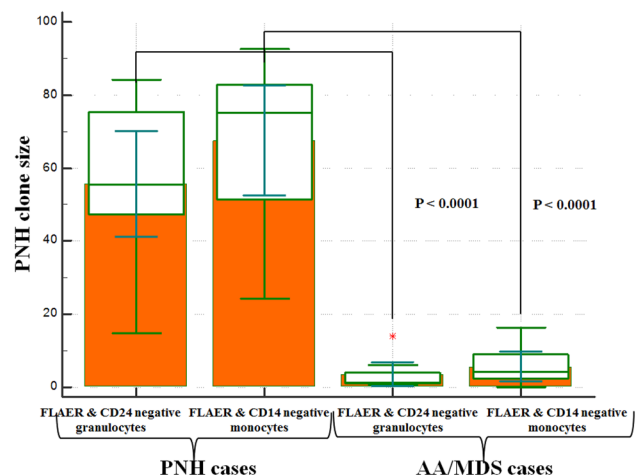


Fig. 3 Box-Whisker plot for representation of clone size by FLAER method between PNH cases and AA/MDS cases

Table 5 Comparisons of PNH clone size in patients with PNH and AA/MDS group

Clone size by FLAER method	PNH (n = 12)	AA/MDS (n = 9)	'p' value
<i>Granulocytes</i>			
< 10%	0	8	0.000044
10–30%	2	1	1.00000
> 30%	10	0	0.000221
<i>Monocytes</i>			
< 10%	0	7	0.000310
10–30%	2	2	1.0000
> 30%	10	0	0.000221

We also observed that clone size on monocytes was always greater than granulocytes in both the groups ($p < 0.0001$). This observation was in agreement with the other studies [10–13]. A single tube FLAER based PNH testing helped in picking up smaller PNH clones associated with AA/MDS patients which were otherwise be missed by non-FLAER based testing. The reason being in CD45 with side scatter alone, one would not be able to provide a pure granulocyte or monocyte gate to detect small populations of GPI-negative cells. The six colour approach would be superior not only because of better GPI-specific reagents, but also due to the fact that the gating reagents allow for a greater purity of granulocytes and monocytes to be analysed [5]. Even in PNH patients, the clone size was slightly higher by using FLAER when compared to non-FLAER based panel. Therefore there was increased sensitivity of PNH clone detection by the former technique. However we did not attempted high sensitivity analysis in this study.

This study had significant positive correlation between FLAER and non-FLAER based test. Initially we were following the stain-lyse-wash-no fix technique for the two tubes non-FLAER based PNH testing (CD45, CD55, CD59, CD66b and CD24) because of fluorochromes overlap among the antibodies. This technique required 5–7 μL of different antibodies, more processing and analysing time, and high cost per test (Rs. 2100/–) to the patient. We followed the recommended method for sample preparation which was lyse-wash-stain-no fix technique [4, 14] for FLAER method that resulted in reduced reagents volume per test (3–5 μL) for various antibodies; also we designed the antibody panel in such a way that all the different fluorochromes were used in a single tube only. Therefore our processing and analysis time and the cost of the test was marginally reduced to Rs. 1800/–. Hence the test efficiency of FLAER based technique is better and in concordance with other studies [10–13]. The recent guidelines [15] published in 2018 were followed up in the subsequent study in our laboratory.

Sutherland et al. [14] recommends that two GPI linked antigens are assessed to confirm PNH on two different lineages, most commonly on RBCs, granulocyte and

monocyte series. The degree of hemolysis correlates better with the percentage of PNH clone on granulocytes and confirm with the monocyte clone size. The testing of RBCs alone is not recommended because of reduced clone size by hemolysis or recent blood transfusion [5, 14, 16]. We did not test for RBC because our PNH cases had significant hemolytic components which might not give appropriate clone size. Some studies [3, 17] also analysed PNH red cells and reticulocytes by using CD59 and glycophorin, and found that the clone size on red cells always lesser than the granulocytes and monocytes either because of hemolysis or dilution due to blood transfusions.

The International PNH interest group (IPIG) [6] divided the patients with PNH clones into three subgroups such as classical PNH, PNH arising in the setting of other bone marrow disorders such as AA and MDS and subclinical PNH (PNH-sc). However, this classification does not address the clinical and laboratory parameters to differentiate these disorders. Hence in this study, we made an attempt to distinguish PNH cases and AA/MDS cases with PNH clones based on simple clinical and laboratory parameters including single tube FLAER-based testing by flow cytometry and it has become 'gold-standard' method nowadays.

Paroxysmal nocturnal hemoglobinuria (PNH) is known for both phenotypic and genotypic heterogeneity [1, 2]. In our study, commonly observed clinical presentations were fatigue (100%), peripheral blood cytopenias (100%), bleeding manifestations (75%), fever (58.3%), abdominal pain (25%) and jaundice (16.6%). These features were very similar to other studies from India [18]. Abdominal pain was considered to be a major risk factor for thrombosis and mortality in PNH patients. Latour et al. [19] reported a higher prevalence of abdominal pain (18.2%) which was comparable to our study. Venous thrombosis was noted in four PNH patients and in all these patients the PNH clone size was > 65% on both granulocytes and monocytes. Few studies have shown a correlation between PNH clone size on granulocytes and the thrombotic risk [19, 20]. Although none of the AA/MDS patients presented with hemoglobinuria, thrombosis or abdominal pain, we did not

find any statistical significant difference in clinical presentation between both the groups because of the smaller cohort size. In contrast, Agarwal et al. [12] found that hemoglobinuria and abdominal pain were seen in PNH cases but not in AA/MDS cases ($p < 0.001$).

Among the various laboratory parameters analysed, only bone marrow cellularity and mean PNH clone size at diagnosis were statistically significant between two groups ($p < 0.05$). The bone marrow was hypoplastic with a cellularity of $< 10\%$ in all aplastic anemia cases. Among MDS cases, the bone marrow was hypocellular in all the three cases with a cellularity of 20–40%. This observation was similar to Wang et al. study [21]. In contrast, 75% of PNH cases showed normal cellularity with erythroid hyperplasia.

On flow cytometry by using FLAER method, majority of the PNH patients (83.3%) had a clone size of $> 30\%$ on both granulocytes and monocytes. Only two patients (16.7%) had 10–30% clone size and none of them had $< 10\%$ clone size. In AA/MDS group, 77.7% patients had $< 10\%$ clone size on both granulocytes and monocytes with remaining 22.3% patients had 10–30% clone size. Our findings were consistent with other reported literature [12, 21]. The major limitation of our study was the smaller sample size in the PNH and AA/MDS cohort and lack of follow-up. The finding of $> 10\%$ clone size in PNH cases and $< 10\%$ clone size in majority of the AA/MDS needs to be validated in much larger studies and follow-up data should substantiate our findings.

Conclusion

A single tube FLAER based testing helped in picking up smaller PNH clones associated with AA/MDS patient which was otherwise missed by two tubes non-FLAER based testing. The latter method had lesser specificity and positive predictive value. Additionally, lyse-wash-stain-no fix and single tube technique for FLAER sample preparation resulted in reduced reagents volume per test (3–5 μL) and cost-benefit to the patient as well (Rs. 1800/- vs. Rs.2100/-). The clone size was $> 30\%$ in majority of PNH cases whereas in AA/MDS, it was usually $< 10\%$ at diagnosis. Even in PNH patients, the clone size was slightly higher by using FLAER when compared to non-FLAER based antibodies panel. Hence we recommend single tube FLAER based PNH testing to be considered ‘gold standard’ cost-effective method because of its improved sensitivity to be done in all suspected PNH, AA and MDS cases; however this needs to be validated in studies with a larger cohort of patients with a good follow-up.

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Compliance with Ethical Standards

Conflict of interest The author declares that they have no conflict of interest.

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