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ORIGINAL ARTICLE

Utilisation of Flow-cytometry in the Diagnosis of Auto Immune Haemolytic Anaemia

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Abstract Auto Immune Haemolytic Anaemia (AIHA) is one of the most common types of acquired haemolytic anaemias. Its main cause is auto-antibody mediated rapid destruction of Red Blood Cells (RBCs). Demonstration of a positive Direct Antiglobulin Test also known as Coomb's test, against these autoantibodies is the crucial serological assay in the diagnosis of AIHA. This routinely used test has the disadvantage of low sensitivity and does not detect low levels of red cell auto antibodies leading to false negative results sometimes. Flow cytometry can effectively diagnose such patients with low levels of autoantibodies. This study was carried out in a tertiary care center, where patients with suspected AIHA were studied during 2 years period. Blood samples of suspected patients of AIHA were tested by both Gel Card Test and by Flow-cytometry for detection of RBC bound IgG. A total of 50 patients with suspected diagnosis of AIHA were studied by flow-cytometry as well as by Gel card test for detection of RBC bound IgG. Out of these 50 cases, 41 cases have turned out to be positive and 9 were negative by flow-cytometry. By Gel card test, out of 50 cases, 34 were positive and 16 were negative. Therefore, there were 7 cases which were negative for RBC bound IgG by Gel card test and these were positive by flow-cytometry. Flow-cytometry is a reliable and more sensitive method and can be used as a new routine diagnostic technique for AIHA.

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Keywords Autoimmune hemolytic anemia (AIHA) · Red blood Cell (RBC) · Flow-cytometry (FCM) · Immunoglobulin · Antibodies

Introduction

Auto Immune Haemolytic Anaemia (AIHA) is one of the most common types of acquired haemolytic anaemias. Its main cause is auto antibody mediated rapid destruction of red blood cells (RBCs) [1]. Detection of these autoantibodies that are directed against erythrocytes is of fundamental importance for diagnosis. A number of techniques have been tested for detection and evaluation of these autoantibodies [2]. Demonstration of a positive Direct Antiglobulin Test (DAT) against these autoantibodies is the crucial serological assay in the diagnosis of AIHA [3]. DAT is also known as Coomb's test and is considered as pathognomonic of immune-mediated haemolysis. This routinely used DAT has the disadvantage of low sensitivity and does not detect low levels of RBC auto antibodies leading to false negative results [4]. Flow cytometry can effectively diagnose such patients with low levels of autoantibodies [5, 6]. Flow-cytometry is gaining importance in several fields of Haematology especially Haemato-oncology. The major advantages of flow-cytometric methods are the speed, the number of parameters which are capable of measurement, the sensitivity, and the ability to quantitate large numbers of cells for a particular criterion. In this study, we investigate the utility of flow cytometry in the diagnosis of patients with suspected AIHA and to detect positivity in patients who are false negative for AIHA by DAT using Gel card Test.

Materials and Methods

This was a prospective study, carried out in a tertiary care center where patients with suspected AIHA were investigated over a 2 year period. Blood samples of suspected patients of AIHA, from Haematology Out Patient Department, were tested by both Gel Card Test and Flow cytometry. The study was approved by the institutional ethics committee. Informed consent from patients was obtained as per guidelines of the institutional ethics committee. For suspecting in vivo hemolysis, inclusion criteria was the presence of three or more of the following four laboratory parameters i.e., (1) Hemoglobin concentration < 8 gm/dl, (2) Reticulocyte count $> 2.5\%$, (3) Total serum bilirubin > 2 mg/dl and (4) Lactate dehydrogenase (LDH) > 500 IU/ml. Exclusion criteria was a history of recent blood transfusion within last 1 month. Peripheral blood of patients was collected in EDTA vacutainer for analysis by Gel Card Test and by Flow cytometry. Flow-cytometric analysis was done by using a FACS canto flow-cytometer (Becton–Dickinson Bio-Sciences Ltd). The software used was FACS Diva. EDTA blood sample of the patients was analyzed for detection and quantification of RBC bound Immunoglobulin G (IgG) antibodies by flow-cytometry. Patient's RBCs were washed three times with normal saline to remove unattached IgG antibodies. 1% suspension of these washed RBCs was prepared with sheath fluid (phosphate buffered saline). Negative Control and positive control was applied with each group of tests in a day. Negative control consisted of RBCs from EDTA blood sample of normal healthy persons having "O" positive blood group. Positive control consisted of RBCs from normal healthy persons having "O" positive blood group sensitized with Anti "D", commonly available in blood banks. RBCs from negative control sample and positive control sample were also washed three times with normal saline to remove unattached IgG antibodies and similarly 1% suspension of these washed RBCs was prepared with sheath fluid (phosphate buffered saline). For flow-cytometry, one stained and one unstained tube was used for each patient and for each negative and positive control. In each of the three unstained tubes, we took corresponding RBCs suspension only. In all the stained tubes, we added FITC conjugated mouse antihuman IgG (BD Biosciences Ltd). After incubation in the dark for 30 min at room temperature, we added fixative 1% formaldehyde solution, vortex and acquired the sample from all these tubes. A minimum of 10,000 events were acquired on flow-cytometer. Relative fluorescent intensity (RFI) of patient, negative control and positive control was noted from the flow-cytometer. Qualitative and quantitative results for diagnosis of AIHA by flow-cytometry were obtained from these RFI values.

Standard procedure as mentioned in the literature of the manufacturer kit was followed for Gel Card test. In a positive test, agglutinated cells formed a red line on the gel surface and a negative result consisted of a compact button of cells formed at the bottom of the microtube.

Results

Clinical Characteristics of the Patients

A total of 50 patients with suspected diagnosis of AIHA were studied by flow-cytometry as well as by Gel card test. The age of the patients ranged from 8 to 73 years with a median age of 44 years. There were 21 males (42%) and 29 (58%) females. At the time of presentation, haemoglobin concentration of these fifty patients varied from 5.5 to 8gm/dl. Reticulocyte count of these fifty patients at the time of presentation ranged from 5.4 to 19.1%. Serum LDH levels of these patients ranged from 527 to 825 IU/ml. All the patients had splenomegaly at the time of presentation.

Interpretation of Results

The mean fluorescent intensity (MFI) of Negative Control was calculated from RFI of ten healthy O group individuals and by taking the mean of these 10 readings of RFI.

Positive cases were defined as having RFI more than or equal to MFI of Negative control + 2 Standard Deviation (SD) of negative control of 10 healthy individuals [4]. Therefore, cases having fluorescence intensity more than or equal to [MFI (Negative Control) + 2 SD] were taken as positive cases for diagnosis of AIHA [4].

This RFI of ten healthy O group individuals ranged from 195 to 247 in our study and the mean of RFI of 10 healthy O group individuals (i.e. MFI Negative Control) was found to be 235 by flow-cytometry. The SD was calculated as 25.31 by statistical work up. The Cutoff point of fluorescent intensity i.e., 235 + 2 SD calculated by statistical work up came out to be 286. The RFI of ten positive controls ranged from 1004 to 1207 and the mean of these 10 positive controls (MFI Positive Control) was calculated to be 1051. The flow-cytometry figures with comparison of population movements, histograms and fluorescent intensity values for one negative and one positive control are shown in Figs. 1 and 2 respectively.

Total 50 suspected cases of AIHA were tested by flow-cytometry and by Gel Card method for detection of RBC bound IgGs. Out of these 50 cases, 41 cases turned out to be positive (having fluorescent intensity ≥ 286) and 9 were negative (having fluorescent intensity < 286) by flow-cytometry. The flow-cytometry figures with comparison of population movements, histograms and fluorescent

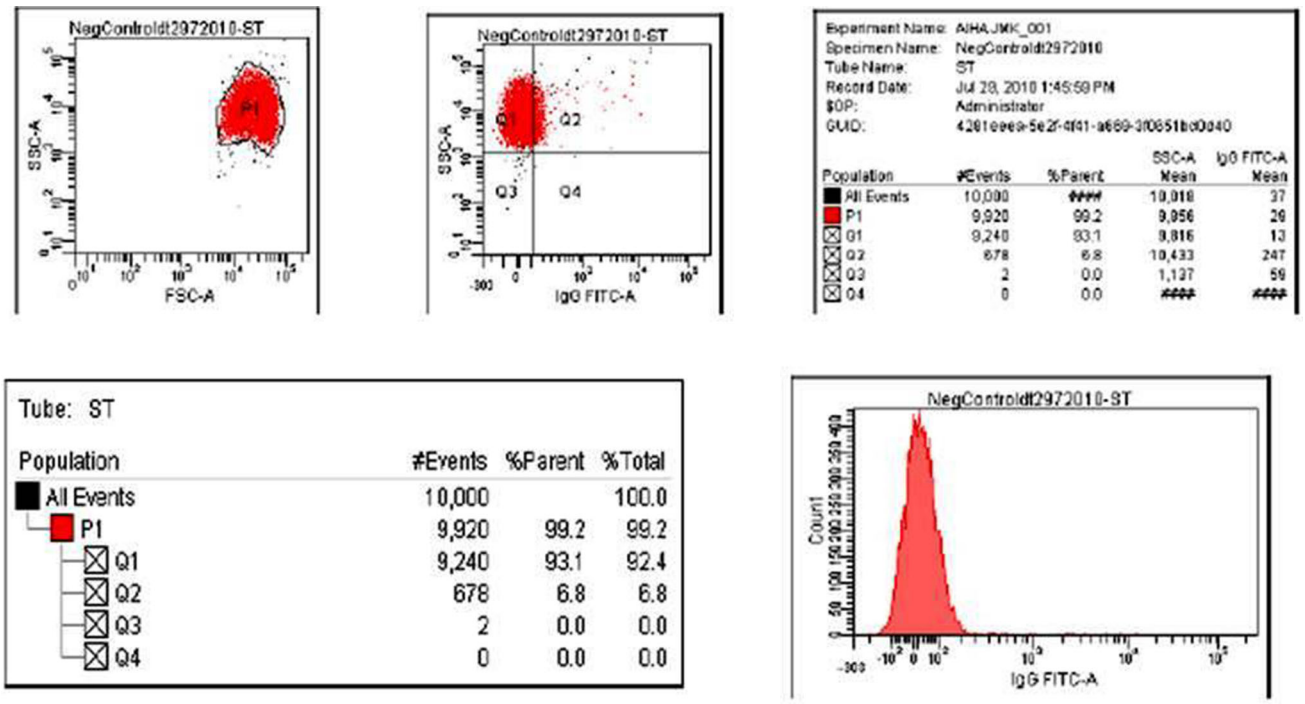

Fig. 1 shows population movements, histograms and fluorescent intensity values for one negative control of our study with small population of reactive cells in Q_2 with fluorescence Intensity of 247 and minimal shift to the right in histogram

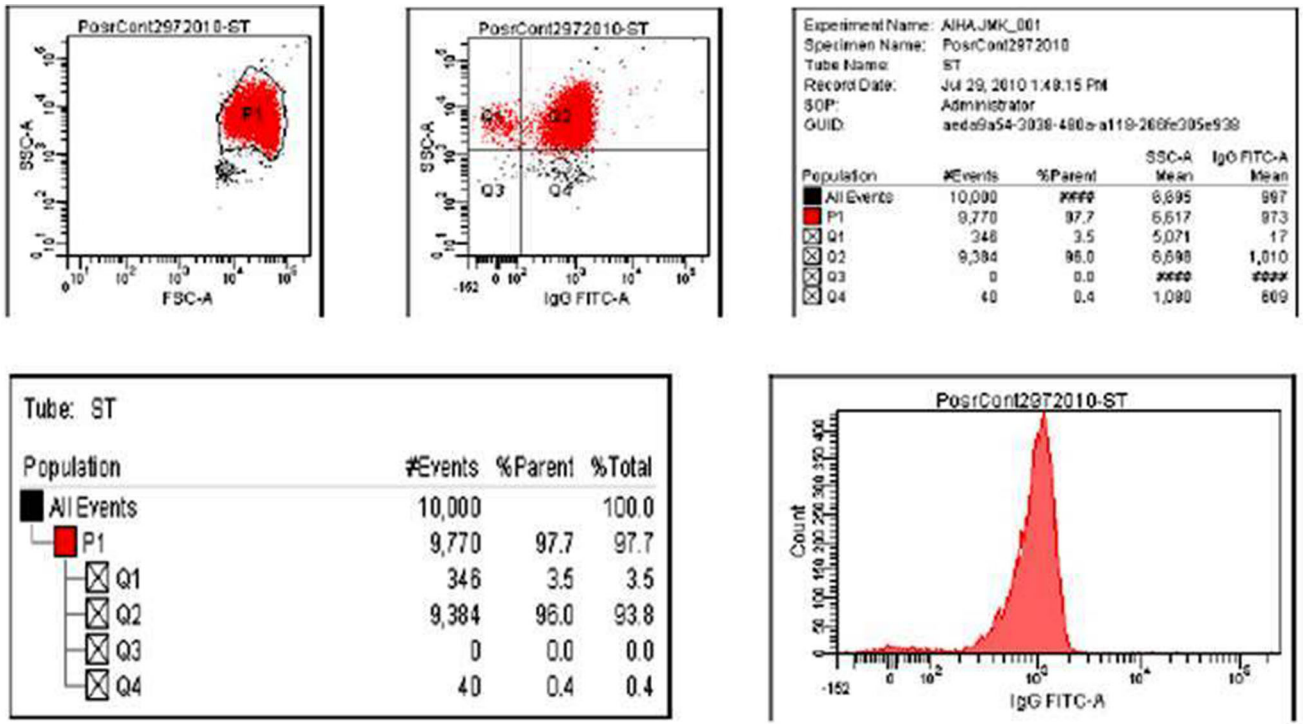

Fig. 2 shows population movements, histograms and fluorescent intensity values for one positive control of our study with majority of population as reactive cells in Q_2 with fluorescence Intensity of 1010 and marked shift to the right in histogram

intensity values for one patient tested negative by flow cytometry and one patient tested positive by flow cytometry is shown in Figs. 3 and 4 respectively.

The same 50 cases were also tested by Gel card test and, out of these 50 cases, 34 were found to be positive and 16 were found to be negative for RBC bound IgG. There were

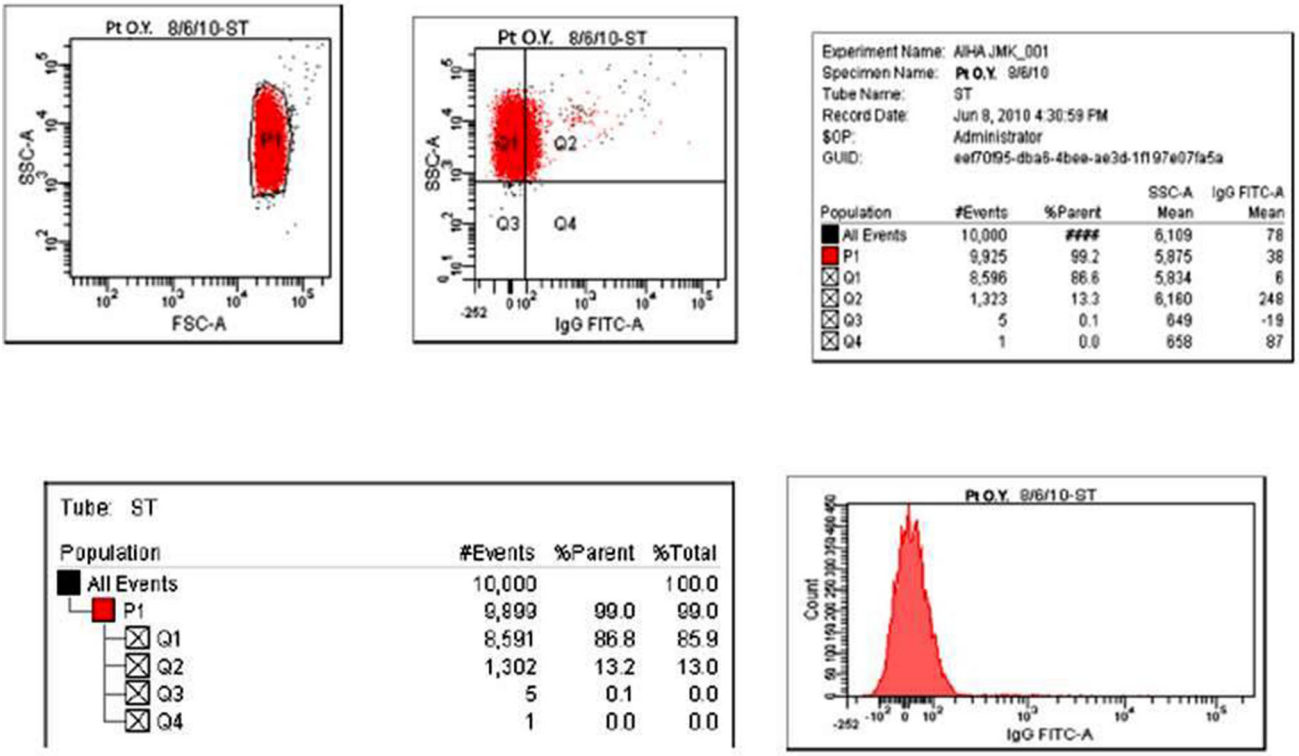

Fig. 3 shows population movements, histograms and fluorescent intensity values for one patient tested negative by flow-cytometry in our study with small population of reactive cells in Q_2 with fluorescence Intensity of 248 and minimal shift to the right in histogram

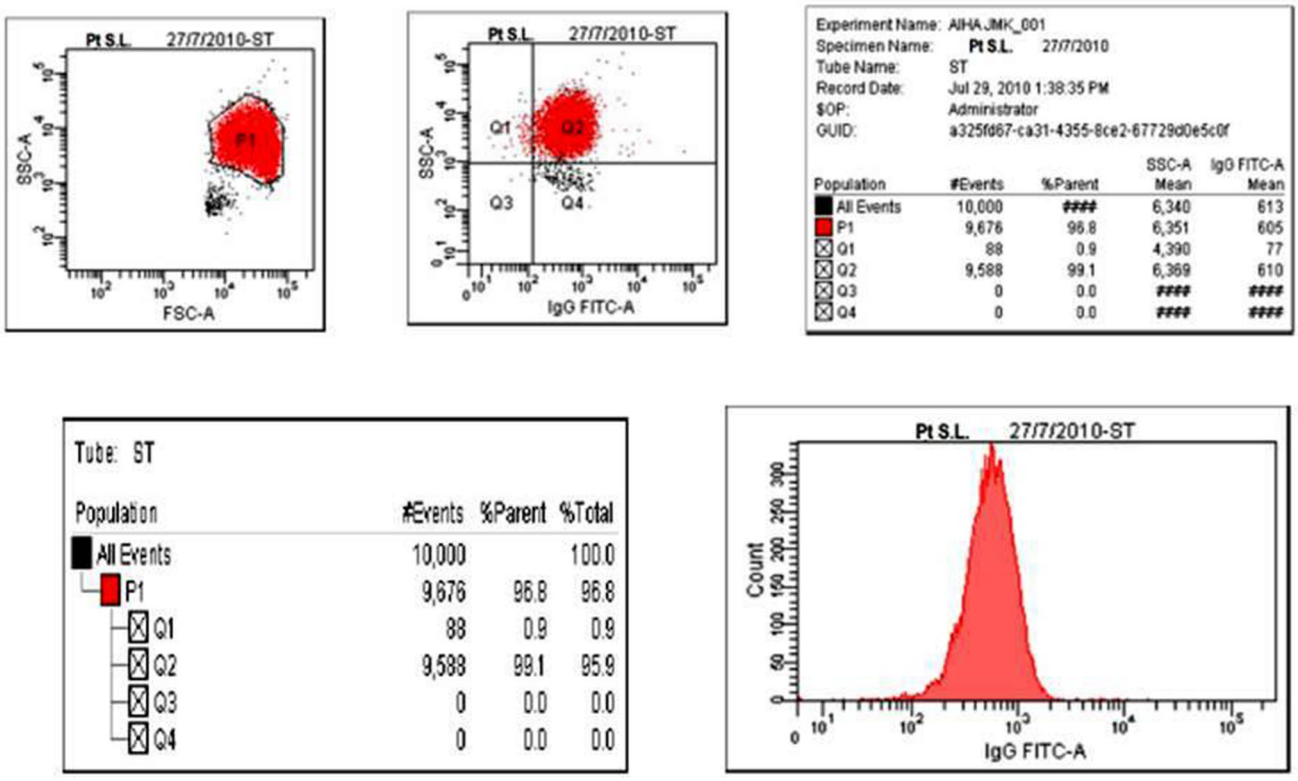

Fig. 4 shows population movements, histograms and fluorescent intensity values for one patient tested positive by flow-cytometry in our study with majority of population as reactive cells in Q_2 with fluorescence Intensity of 610 and significant shift to the right in histogram

7 cases which were negative for RBC bound IgG by Gel card test but were positive for RBC bound IgG by flow-cytometry.

Quantitative results: The quantification of positivity by flow-cytometry is obtained by calculating percentage fluorescence. The calculation of percentage fluorescence is a standard technique originally described by Vander Meulen et al. [7] and is still being followed. It is calculated by formula as given below

$$\% \text{ fluorescence} = \frac{\text{RFI (Pts red cells)} - \text{MFI (negative control)}}{\text{MFI (positive control)} - \text{MFI (negative control)}} \times 100$$

Using percentage fluorescence, the relative amount of IgG antibody, present on RBCs of different patients of AIHA, was compared. From the value of our Cut off fluorescent intensity of 286, the cut off percentage fluorescence in our study was calculated to be 6.17%.

Also, percentage fluorescence was calculated for all the 50 cases. The percentage fluorescence of our negative cases ranged from 0.61 to 2.7% with a mean of 2.08%. The percentage fluorescence of our positive cases ranged from 33.58 to 67.77% with a mean of 52.57%. The percentage fluorescence of 7 cases which were negative by Gel card test (GT) but positive by flow-cytometry ranged from 30.27 to 38.23% with a mean of 36.41%. These comparative results of Coomb's test by Gel Card and by flow-cytometry in 50 suspected cases of AIHA in our study are given in the tabulated form in Table 1.

Discussion

In this study, we detected the presence of RBC bound IgG antibodies by flow-cytometry in suspected cases of AIHA and the same cases were subjected to DAT by Gel card test. In our study, we found that there were seven out of 50 cases which were negative by Gel card test but positive by flow-cytometry method.

Direct Antiglobulin Test by conventional tube technique is the most popular method practiced in blood centers for diagnosis of AIHA [8]. A positive DAT is generally regarded as indicative of a diagnosis of AIHA. However, a negative DAT does not rule out the diagnosis of AIHA [9]. These patients identified as Coombs negative AIHA possibly carry a lower number of IgG molecules per RBC, and concurring a negative tube DAT [10]. The Coomb's Tube Test (CTT) can effectively diagnose AIHA only when > 500 molecules of auto antibodies are bound per RBC [11, 12]. AIHA at times is also missed by Gel card Test as it requires approximately 200 molecules of IgG per RBC for a positive DAT [13]. A negative DAT usually excludes auto immune haemolytic anaemia. However, in the literature, it is mentioned that in 2% of warm AIHA, this DAT can be negative [14]. There are studies in which the incidence of DAT negative AIHA was detected more frequently [2, 4]. The incidence of DAT negative AIHA in one study [2] was found to be 10%. In another study [4] the incidence was 37% (3 out of 8 cases). In our study, the incidence of DAT negative AIHA was found to be 14% (7 out of 50 cases). Flow cytometry is the most sensitive of all the antibody detection techniques with a potential to detect as low as 30–40 molecules of immunoglobulins per RBC [15].

There are several explanations which have been given in this regard. The most important explanation being given is low level of sensitising red cell autoantibody, which is below the sensitivity of the test being employed, e.g. Coomb's Tube Test, Gel card test [16].

Other explanations given are presence of IgA or other immunoglobulins coating the RBCs [16] or involvement of low affinity antibodies that may easily dissociate during washing of RBCs [17]. Therefore, more sensitive technique such as Flow-cytometry is now being increasingly used by immune-hematologists to characterize red cell bound immunoglobulins because of higher accuracy, reproducibility and sensitivity [12]. In one study, the authors have mentioned that in their centre, they are using CTT and Gel card test to evaluate DAT positive patients and flow cytometry is being used only in special circumstances such as negative DAT with clinical suspicion of AIHA [2]. In the management of DAT-negative hemolytic anaemia, as per a study in the year 2009, it is important to distinguish Coombs-negative AIHA patients from other hemolytic anaemia [18]. This is because steroid treatment has major effects on AIHA [19]. Steroids have also been associated with several serious side effects, which makes clinicians hesitate to use steroids to treat DAT-negative haemolytic anaemia patients without diagnosis of AIHA [20].

Table 1 Shows the comparison of results by Gel Card test (GT) & flow-cytometry (FCM) in 50 suspected cases of AIHA in our study

Suspected AIHA cases (N = 50)	Coombs test (gel card)	Flow-cytometry	Mean fluorescent intensity (Range)	Mean percentage fluorescence (Range)
34 Cases	Positive	Positive	664 (509–788)	52.57% (33.58–67.77%)
7 Cases	Negative	Positive	532 (482–547)	36.41% (30.27–38.23%)
9 Cases	Negative	Negative	252 (240–257)	2.08% (0.61–2.7%)

In our study, we have used percentage fluorescence, as a measure of quantification of RBC bound IgG. In another study, the percentage fluorescence by flow-cytometry was used to assess diagnosis in CTT-DAT-negative AIHA [21]. It was proposed in their study that flow cytometry percent fluorescence cut-off values should be employed to determine the Coombs' negative AIHA cases. This study was performed to assess the role of gel test and flow cytometry in diagnosis of Coombs' negative AIHA. It was also concluded in this study that flow cytometry is more sensitive than Gel card test for assessing CTT-DAT-negative AIHA.

There are studies that have used immune-radiometric assay (IRMA) to detect RBC-IgG quantitatively [22]. In another study [18], where IRMA was used, the authors suggested that, although the simple methods are desired, quantitative measurements should be used to guarantee their ability to measure sub threshold IgG in cases of AIHA. The normal value of RBC-IgG that is number of IgG molecules per RBC in 100 healthy Japanese adults, has been reported [23] as (33 ± 13). There are other studies [22, 24] in which very similar values have been reported. A direct correlation between the number of molecules of autoantibodies IgG per RBC and in vivo hemolysis has been reported [25]. There are studies [26, 27] in which, the usefulness of quantification and measurement of RBC-IgG in the diagnosis for Coombs-negative AIHA have been reported. Several studies [27, 28] have reported patients with DAT negative AIHA, in which the number of molecules of IgG were below the sensitivity of the test being employed for DAT. In one study [29], patients with Coombs-negative AIHA were reported to have abnormal levels of IgG, ranging from 70 to 434 molecules of IgG/RBC. It was proposed in one study [18] to adopt the clinical diagnosis as the gold standard for the diagnosis of Coombs-negative AIHA, as many clinicians have previously clinically diagnosed Coombs-negative AIHA by the presence of haemolysis and responsiveness to steroid treatments with respect of value of IgG molecules/RBC. Another study was conducted in year 2015, with an aim to develop a flow cytometry protocol for simultaneous detection of IgG, IgM, IgA immune complexes and C3d attached to RBCs in AIHA patients [30]. They used antibodies conjugated with different fluorochromes such as IgG-FITC, IgM-PE and IgA-PerCP for AIHA patients in their study [30].

Conclusion

Flow-cytometry is a reliable and sensitive method of detecting RBC-bound IgG antibodies for the diagnosis of AIHA. This can be used in those suspected AIHA cases where gel card test is negative.

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