Original article

Detection of red blood cell-bound antibody by flow cytometry as compared with direct antiglobulin test in patients with autoimmune hemolytic anemia (AIHA)

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Abstract:

Introduction: *Autoimmune hemolytic anemia (AIHA) is a condition in which the body produces antibodies against red blood cells, leading to their destruction. Diagnosis relies on clinical symptoms, laboratory evidence of hemolysis, and the detection of antibodies on red blood cells. However, there are some patients with AIHA who lack detectable antibodies, this leads to diagnostic challenges. Based upon the fact that flow cytometry can detect low level of IgG on cells, the chance to detect patients with AIHA is potentially increased.* ***Objective:*** *To compare the diagnostic capabilities of flow cytometry versus the current method of direct antiglobulin test (DAT) in diagnosing AIHA.* ***Materials and Methods:*** *DAT is performed using column agglutination technology (CAT) with polyspecific Coombs' reagent, capable of detecting both IgG and C3d. Flow cytometry is performed using reagents that can only detect IgG, and negative control testing is conducted each time. Blood samples from healthy individuals were studied to establish a cut-off for flow cytometry. Then blood samples from patients who underwent DAT were examined using flow cytometry. Patients with laboratory evidence of hemolysis were further tested to identify the underlying cause.* ***Results:*** *The cut-off value for flow cytometry was determined from 41 healthy participants. In the second phase, 44 out of 185 had AIHA. Flow cytometry showed 90.9% sensitivity (95%CI: 78.3-97.5) and 66.0% specificity (57.5-73.7). DAT showed 88.6% sensitivity (75.4-96.2) and 61.7% specificity (53.2-69.8). Flow cytometry aids in diagnosing 5 cases of low-level IgG-mediated AIHA but yields negative results in 2 cases of IgM-mediated AIHA and 2 cases of low-affinity IgG-mediated AIHA.* ***Conclusion:*** *Flow cytometry can be used to aid in the diagnosis of AIHA with good sensitivity and specificity. It also assists in diagnosing DAT negative AIHA caused by low levels of IgG but has limitations in detecting other type of antibodies and low-affinity IgG. Using flow cytometry in conjunction with DAT can enhance the sensitivity of AIHA detection.*

Keywords : ● *Autoimmune hemolytic anemia* ● *Direct antiglobulin test* ● *Direct Coombs' test* ● *Flow cytometry*

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นิพนธ์ต้นฉบับ

การตรวจหาแอนติบอดีบนผิวเม็ดเลือดแดงในผู้ป่วยโรค autoimmune hemolytic anemia (AIHA) โดยวิธี flow cytometry เปรียบเทียบกับวิธี direct antiglobulin test

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บทคัดย่อ

บทนำ *Autoimmune hemolytic anemia (AIHA) เป็นภาวะที่ร่างกายสร้างแอนติบอดีต่อเม็ดเลือดแดงทำให้เกิดการทำลายเม็ดเลือดแดงขึ้น การวินิจฉัยภาวะนี้อาศัยอาการทางคลินิก ผลตรวจทางห้องปฏิบัติการพบหลักฐานการแตกของเม็ดเลือดแดง และการตรวจพบแอนติบอดีบนผิวเม็ดเลือดแดง อย่างไรก็ตามมีผู้ป่วย AIHA บางรายที่ตรวจไม่พบแอนติบอดีบนผิวเม็ดเลือดแดงทำให้เกิดความลำบากในการวินิจฉัย flow cytometry เป็นวิธีการตรวจที่สามารถพบแอนติบอดีชนิด IgG ปริมาณน้อยบนผิวเซลล์ได้จึงอาจช่วยเพิ่มความสามารถในการวินิจฉัยผู้ป่วย AIHA* ***วัตถุประสงค์*** *เพื่อเปรียบเทียบความสามารถในการวินิจฉัยภาวะ AIHA ระหว่างการตรวจด้วยวิธี flow cytometry และวิธี direct antiglobulin test (DAT)* ***วัสดุและวิธีการ*** *DAT ตรวจโดย column agglutination technology (CAT) ซึ่งใช้น้ำยา Coombs' reagent แบบหลายความจำเพาะที่สามารถตรวจพบได้ทั้ง IgG และ C3d ส่วน flow cytometry ตรวจโดยใช้น้ำยาที่สามารถตรวจพบได้เฉพาะ IgG และมีการตรวจตัวอย่างควบคุมแบบลบพร้อมกันทุกครั้ง เกณฑ์ตัดสินผลบวกของ flow cytometry ได้จากการตรวจตัวอย่างเลือดจากผู้ที่มีสุขภาพดี หลังจากนั้นตัวอย่างเลือดจากผู้ป่วยที่ได้รับการส่งตรวจ DAT จะได้รับการตรวจด้วยวิธี flow cytometry ด้วย ผู้ป่วยที่มีหลักฐานการแตกของเม็ดเลือดแดงจะได้รับการตรวจเพิ่มเติมเพื่อหาสาเหตุต่อไป* ***ผลการศึกษา*** *เกณฑ์การตัดสินผลบวกของ flow cytometry ได้จากผู้ที่มีสุขภาพดี 41 ราย ในระยะที่สองของการศึกษาจากผู้ป่วยทั้งหมด 185 ราย มี 44 รายที่มีภาวะ AIHA การตรวจโดยวิธี flow cytometry มีความไวร้อยละ 90.9 (95%CI: 78.3-97.5) และความจำเพาะร้อยละ 66.0 (57.5-73.7) ส่วนวิธี DAT มีความไวร้อยละ 88.6 (75.4-96.2) และความจำเพาะร้อยละ 61.7 (53.2-69.8) พบว่า flow cytometry ช่วยในการวินิจฉัยผู้ป่วย AIHA ได้เพิ่มเติม 5 รายซึ่งเกิดจาก IgG ปริมาณน้อย แต่ให้ผลลบในผู้ป่วย AIHA ที่เกิดจาก IgM 2 ราย และ low-affinity IgG 2 ราย* ***สรุป*** *Flow cytometry สามารถใช้ในการวินิจฉัย AIHA ได้โดยมีความไวและความจำเพาะที่ดี นอกจากนี้ยังช่วยในการวินิจฉัยผู้ป่วย AIHA ที่ให้ผลลบกับการตรวจ DAT เนื่องจากปริมาณ IgG ที่น้อยเกินไปได้ด้วย ทั้งนี้ flow cytometry ก็มีข้อจำกัดคือไม่สามารถตรวจพบแอนติบอดีชนิดอื่น หรือ low-affinity IgG การใช้ flow cytometry ร่วมกับ DAT สามารถช่วยเพิ่มความไวในการวินิจฉัย AIHA ได้*

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Introduction

Autoimmune hemolytic anemia (AIHA) is a condition in which the body produces antibodies against red blood cells, leading to their destruction. The incidence is approximately 1 case per 75,000-85,000 individuals per year, with a higher incidence in people aged over 40 years[1]. Approximately 75% of patients diagnosed with AIHA are warm AIHA, which occurs when the body produces antibodies against red blood cells that react at 37 degrees Celsius and are often immunoglobulin G (IgG). The remaining 15% are diagnosed with cold AIHA, and 5-10% have mixed type AIHA[2].

The diagnosis of AIHA relies on clinical symptoms, laboratory evidence of hemolysis, and the detection of antibodies on red blood cells[3,4]. A commonly used method to detect antibodies or complements attached to red blood cells is the direct Coombs' test, also called direct antiglobulin test (DAT). This test involves the use of Coombs' reagent (antihuman globulin; AHG) that reacts with antibodies or complements on red blood cells. The reaction results in the agglutination of red blood cells, and a positive result is reported when agglutination occurs. The DAT can be performed using the conventional tube test (CTT) or the column agglutination technology (CAT). However, it has been observed that some patients exhibit clinical symptoms and laboratory findings consistent with AIHA, despite having a negative DAT result. These patients are referred to as Coombs' negative AIHA patients, which accounts for 2-11% of individuals diagnosed with AIHA.[5-7] This is caused by[7-10]:

- The quantity of antibodies on red blood cells is too low.
- Antibodies attached to red blood cells with low affinity, making them easily washed off during the cell-washing process.
- Types of antibodies on red blood cells are not detected by DAT, such as non-complement-fixing IgM and IgA.

Previous studies have found that the detection of antibodies on red blood cells using the DAT requires a minimum of 100-200 molecules per cell[11,12]. By contrast, flow cytometry is a technique which detects antibodies on cell surfaces by using fluorescence, which specifically binds to antibodies, then measures light signals by the analyzer, with the ability to detect as few as 30-40 molecules per cell[11,12]. Since flow cytometry offers greater analytic sensitivity, it may help reducing discrepancies between the detection of antibodies on red blood cells, clinical symptoms, and other laboratory tests. This assists healthcare professionals in making more informed and appropriate treatment decisions.

This study aims to compare the diagnostic sensitivity and specificity of the DAT with flow cytometry in diagnosing AIHA.

Materials and Methods

Currently, the King Chulalongkorn Memorial Hospital (KCMH) blood bank conducts the detection of antibodies on red blood cells using the DAT method via CAT. This method does not involve a pre-washing step for red blood cells. The test begins with a gel card containing polyspecific Coombs' reagent (ID-LISS/Coombs card, Bio-Rad Laboratories, California, USA), capable of detecting both IgG and C3d but unable to differentiate between them. If the test yields a positive result, a further test is performed using a gel card containing monospecific Coombs' reagent (ID-card DC screening II, Bio-Rad Laboratories, California, USA), which can differentiate whether the positive result is due to IgG or C3d.

The flow cytometry test utilizes a method adapted from the procedure outlined by Thedsawad et al.[8] It involves using mouse IgG1 negative control: FITC (Bio-Rad, California, USA) solution for the negative control tube and mouse antihuman IgG (Fc) CH2 domain: FITC (Bio-Rad, California, USA) solution for the tube intended to detect antibodies on the surface of red blood cells. Subsequently, the samples are analyzed using the BD FACSCanto II instrument (Becton, Dickinson and Company, New Jersey, USA), and the results are interpreted using the BD FACSDiva Software program (Becton, Dickinson and Company, New Jersey, USA).

This research is divided into two phases.

1. Determination of reference values for flow cytometric analysis

The first phase involves obtaining reference values for mean fluorescence intensity (MFI) through flow cytometry, utilizing residual specimens from individuals who visited the check-up clinic at KCMH. These individuals had undergone a complete blood count (CBC) test, and their results were within the normal range. Following this, their specimens were tested using the DAT, and only those with negative results proceeded to flow cytometry testing.

2. Comparison of the performance of flow cytometry and DAT to detect AIHA

After obtaining the MFI reference values, the second phase involves testing specimens from patients receiving treatment at KCMH, which were sent for DAT, using flow cytometry. This aims to compare the diagnostic capabilities of both methods by evaluating the results against other laboratory tests. If hemolysis markers are found in at least 3 out of the following 6 criteria, a diagnosis of hemolysis will be established (tests conducted before and after the DAT within 7 days): hemoglobin < 12 g/dL, reticulocyte count > 2%, lactate dehydrogenase (LDH) > 220 U/L, total bilirubin > 1.2 mg/dL, haptoglobin < 25 mg/dL, and presence of microspherocyte from peripheral blood smear.

The results will be interpreted or further tested as illustrated in Figure 1. In the second phase, every time flow cytometry is conducted, a negative control is concurrently performed using red blood cell products from the National Blood Centre, Thai Red Cross Society, which are less than 7 days old from the collection date. These blood products are donated by healthy individuals and have undergone DAT testing with negative results. The negative control samples undergo the same process as patient samples and are tested by flow cytometry simultaneously. The obtained MFI value from negative control must be lower than the cut-off value to interpret the patient's test results.

Exclusion criteria

- Sample from patients younger than 15 years old (the reference values obtained from the check-up clinic are derived from individuals aged 15 and above).
- Samples from the same patients who have previously undergone testing in this study.
- The quantity of the specimen is insufficient for analysis using flow cytometry.
- The specimen has been collected for more than 24 hours prior to analysis.

Sample size

- For obtaining reference values: 40 samples.
- For comparison between flow cytometry and DAT:

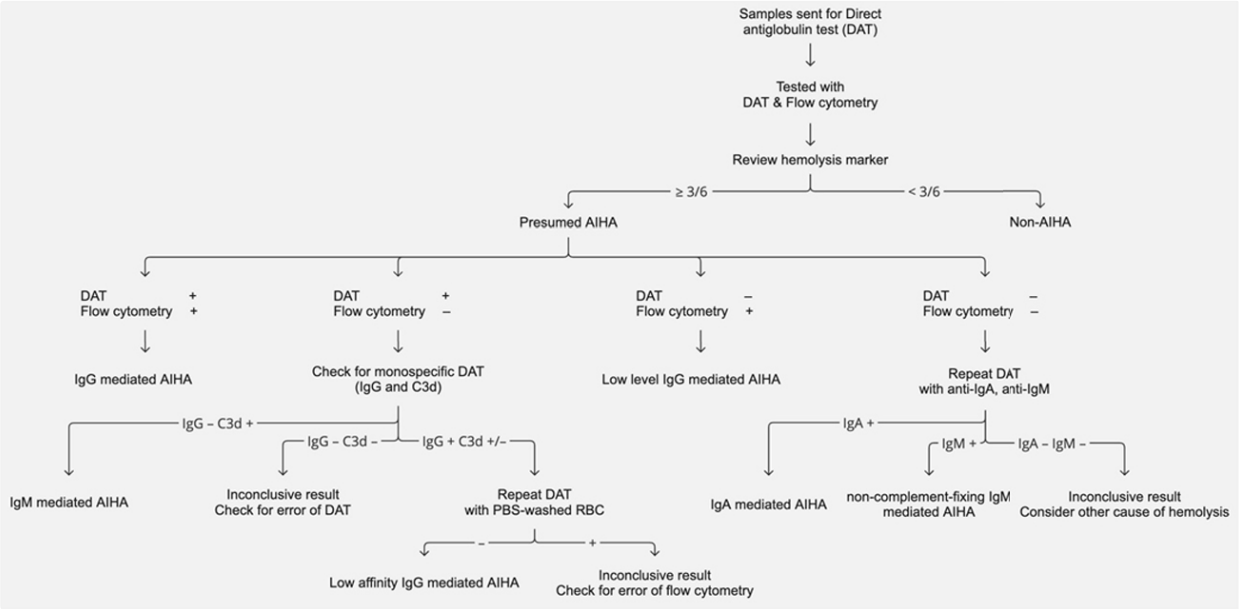

Figure 1 The steps of analysis, interpretation, and additional tests in this research

Calculated from the formula $\frac{Z^2P(1-P)}{d^2}$, with the specified values: p (expected sensitivity) = 0.9, d (allowable error) = 0.1, α = 0.05 which gives Z = 1.96. The result is n = 34.57.

From data collected at the KCMH blood bank between June and December 2020, it was found that approximately 15% of individuals who underwent the DAT were diagnosed with AIHA. Therefore, for this research project, a planned sample size of approximately 240 individuals is intended. This number may vary depending on the prevalence of the disease, aiming to ensure 35 cases of AIHA.

Statistical method

All data were analyzed using the Statistical Package for Social Sciences (SPSS) software. The characteristics of the sample group used to establish reference values and the sample group used for comparison between methods were described using descriptive statistics. The 95th percentile of MFI from the sample group used to establish reference values was found and this value was then used as a cut-off to interpret positive results in the next analysis step. The results of flow cytometry and DAT in diagnosing AIHA were compared in the form of a 2x2 table and the diagnostic sensitivity, diagnostic specificity, positive predictive value (PPV), and negative predictive value (NPV) were presented. The diagnostic capabilities for AIHA between flow cytometry and DAT were compared using Cohen's kappa statistic. The MFI values from flow cytometry and the grade of reaction from DAT were compared using Spearman's rank correlation. The statistical significance level was set at a *p*-value < 0.05.

Results

Clinical characteristics

In the first phase of the research, there were a total of 41 participants whose samples were collected during December 2022 to January 2023, ranging in age from 16 to 82 years, with a median age of 54 years. The participants included 13 males (31.7%) and 28 females (68.3%). The average hemoglobin level for males was 15.3 g/dL, and for females was 13.2 g/dL. All participants had negative DAT results. In the second phase, there were a total of 185 participants whose samples were collected during February 2023 to November 2023, comprising 71 males (38.4%) and 114 females (61.6%). The participants ranged in age from 18 to 100 years, with a median age of 64 years.

Interpretation of results

The flow cytometry MFI results from the first phase showed a right-skewed distribution, with a mean of 8.51 and a median of 8. The 95th percentile was calculated using non-parametric methods and found to be 15.

In the second phase, DAT that yielded results from weakly positive and above were interpreted as positive. For flow cytometry, the test was analyzed twice per participant (including negative controls), and the average MFI was calculated. If the average MFI was 15 or above, it was interpreted as positive. Using the hemolysis marker criteria of at least 3 out of 6 criteria as described in the materials and methods, along with the detection of antibodies on red blood cells using either flow cytometry or DAT as the gold standard for diagnosing AIHA, it was found that 44 participants received a diagnosis of AIHA. The diagnostic capabilities for AIHA using flow cytometry and DAT are compared in Table 1. It can be observed that flow cytometry has a sensitivity of 90.9% (95%CI: 78.3-97.5%), specificity of 66.0% (95%CI: 57.5-73.7%), PPV of 45.5% (95%CI: 39.4-51.6%), and NPV of 95.9% (95%CI: 90.1-98.4%), while DAT has a sensitivity of 88.6% (95%CI: 75.4-96.2%), specificity of 61.7% (95%CI: 53.2-69.8%), PPV of 41.9% (95%CI: 36.4-47.7%), and NPV of 94.6% (95%CI: 88.3-97.6%). The Cohen's kappa statistic was tested to determine the agreement between the two testing methods, and it was found to have a moderate level of agreement according to the interpretation by McHugh ML[13]. The statistical significance was observed (*Kappa* = 0.773, $p < 0.001$). The Spearman's rank correlation was run to find the relationship between MFI values from flow cytometry and grades of reaction from DAT. There was a strong positive correlation[14] with a statistical

Table 1 Comparison of diagnostic capabilities for AIHA using flow cytometry and DAT

		AIHA	non-AIHA
Flow cytometry	positive	40	48
	negative	4	93
DAT	positive	39	54
	negative	5	87

AIHA = autoimmune hemolytic anemia; DAT = direct antiglobulin test

significance (*Spearman's rank correlation coefficient* = 0.793, $p < 0.001$).

Among the patients meeting the hemolysis diagnostic criteria (hemolysis markers ≥ 3), a total of 76 cases, 35 showed consistently positive results in both flow cytometry and DAT, while 32 exhibited consistently negative results in both methods. Upon further testing, no cases yielded positive results in DAT when tested with anti-IgA and anti-IgM reagents. Among the patients with discrepant results between the two methods, 5 showed positive results in flow cytometry but negative results in DAT, and 4 exhibited negative results in flow cytometry but positive results in DAT. Subsequent testing of these flow cytometry-negative/DAT-positive cases revealed that 2 reacted with monospecific Coombs' reagent specific to anti-C3d but not with anti-IgG. In the other 2 cases, when red blood cells from these patients were washed with room temperature phosphate-buffered saline solution 7 times (equivalent to the number of washes in the flow cytometry testing process), the DAT results turned negative.

In the group with hemolysis markers less than 3, there were a total of 109 cases. In this group, 70 individuals did not undergo testing for the complete set of 5 markers (microspherocyte from peripheral blood smear does not require testing order; if found, the laboratory will automatically report it). Both methods gave concordant positive results for 45 cases and concordant negative results for 52 cases. Flow cytometry yielded positive results while DAT gave negative results for 3 cases, and flow cytometry yielded negative results while DAT gave positive results for 9 cases. All cases with discrepant results between the two methods are summarized in Table 2.

Discussion

The findings of this study demonstrated that flow cytometry can be used to make the diagnosis of AIHA with good diagnostic sensitivity and specificity. It is noteworthy that the prevalence observed in this study, derived from patients' samples sent for DAT testing, tends to be higher than normal due to pretest suspicion of hemolysis. Additionally, some patients who did not meet the criteria for hemolysis (hemolysis markers < 3) underwent incomplete testing, potentially leading to the misclassification of patients with actual hemolysis into the non-hemolysis group in this research. Therefore, the interpretation of PPV and NPV, which are influenced by prevalence, may not be practically applicable. However, focusing on sensitivity and specificity alone revealed that flow cytometry has both better sensitivity and specificity than DAT, though the statistical significance of this difference was not established.

Considering cases with discrepant results between the two methods, it was found that flow cytometry aided in the diagnosis of AIHA for 5 cases out of a total of 44 cases. This aligns with previous studies that approximately 2-11% of all AIHA patients are Coombs' negative AIHA[5–7]. Notably, there were cases with negative results in flow cytometry but positive results in DAT, totaling 4 cases, resulting from IgM-mediated AIHA (2 cases) and low-affinity IgG (2 cases). This highlights the interesting observation that the challenge of being unable to detect low-affinity IgG, typically discussed

Table 2 Summary of cases with discrepant result between flow cytometry and DAT

Hemolysis marker ≥ 3	FC	DAT	Case	Sex	Age	MFI	DAT grade			Conclusion
							Polyspecific	anti-IgG	anti-C3d	
Yes	Negative	Positive	C18	M	68	9.5	3+	0	4+	IgM-mediated AIHA
			C33	F	84	12.5	1+	0	1+	IgM-mediated AIHA
			C136	F	45	11	w+	w+	0	Low-affinity IgG-mediated AIHA
			C190	F	61	9.5	1+	1+	0	Low-affinity IgG-mediated AIHA
	Positive	Negative	C47	F	55	21	0			Low-level IgG-mediated AIHA
			C78	M	61	19	0			Low-level IgG-mediated AIHA
			C104	M	56	26	0			Low-level IgG-mediated AIHA
			C135	F	82	19	0			Low-level IgG-mediated AIHA
			C197	F	27	21	0			Low-level IgG-mediated AIHA
No	Negative	Positive	C4	F	60	6	1+	1+	0	N/A, NCHW
			C7	M	63	10.5	1+	0	1+	IgM-coated RBC
			C48	F	57	11.5	1+	1+	0	N/A
			C141	F	81	13	1+	1+	0	N/A
			C148	M	65	10.5	w+	w+	0	N/A
			C151	M	22	13.5	w+	w+	0	N/A, NCHW
			C154	F	49	8.5	1+	1+	0	N/A, NCHW
			C188	F	67	14.5	w+	w+	0	N/A
			C195	F	30	12	1+	1+	0	N/A
	Positive	Negative	C51	F	73	24	0			N/A, NCHW
			C57	M	81	18.5	0			N/A, NCHW
			C131	M	88	15	0			N/A, NCHW

AIHA = autoimmune hemolytic anemia; DAT= direct antiglobulin test; F = female; FC = flow cytometry; M = male; MFI = mean fluorescence intensity; N/A = no answer; NCHW = not complete hemolysis work-up

in the context of DAT methods that require red blood cell washing before testing[5,6,10], is also encountered in flow cytometry, which involves multiple red blood cell washes before analysis.

A previous study by Kamesaki and Kajii[15] found that the causes of Coombs' negative AIHA were approximately 80% due to the level of IgG under the detection threshold, about 15% due to low-affinity IgG, and around 5% due to red blood cell-bound IgA or non-complement-fixing IgM. In our present study, however, we did not identify any AIHA patients resulting from non-complement-fixing IgM or IgA.

Furthermore, examining patients who did not meet hemolysis criteria revealed that flow cytometry could detect antibodies on red blood cells in an additional 3 cases compared to DAT. Conversely, there were cases where DAT detected antibodies on red blood cells while flow cytometry yielded negative results for 9 cases. Notably, 1 case could be explained by the antibody type (IgM), while the causes in the remaining 8 cases were not explored. Interestingly, when considering other laboratory results with clinical diagnoses, it was found that out of these 8 patients, 6 had underproduction anemia. This may be explained by the fact that in these conditions, there is a prolonged red blood cell lifespan, leading to a higher presence of senescent red blood cells in circulation. These senescent cells express senescent antigens and can be recognized by naturally occurring IgG, which is one of the mechanisms the body employs to eliminate aged red blood cells[16-19]. The binding between senescent antigens and natural occurring IgG has low affinity, which may account for the observed discrepancies between flow cytometry and DAT in these cases.

Based on the findings of this study, the authors propose the utilization of flow cytometry as an additional diagnostic tool in situations where DAT produces negative results, yet the patient presents significant clinical suspicion of AIHA. However, it is noteworthy that flow cytometry has limitations that may lead to false negatives, such as when the antibody type is not detected by the flow cytometry reagents or when the antibody has low affinity and is lost during the cell-washing process. Consequently, it is advisable not to solely depend on flow cytometry as an independent diagnostic test for AIHA.

This research has certain limitations, including the following: participant selection relying on clinicians' orders for DAT testing might not accurately represent the true prevalence in patients; the diagnostic criteria for AIHA relied solely on laboratory test results and did not consider clinical presentations; the response to treatment had not been assessed; some patients in the non-hemolysis group might not have undergone complete hemolysis marker testing, leading to potential misclassification; patients in the non-hemolysis group were not further investigated, so the causes of discrepant results could not be determined. If further studies are to be conducted, the authors suggest validating the flow cytometry process using a red blood cell washing method that prevents the loss of low-affinity antibodies, such as maintaining the temperature at 4 degrees Celsius throughout the washing process[10,20,21]. This may help mitigate limitations in detecting low-affinity antibodies.

Conclusion

This research demonstrates that flow cytometry can be used to aid in the diagnosis of AIHA with good sensitivity and specificity. It also assists in diagnosing DAT negative AIHA caused by low levels of IgG, which can be beneficial for making treatment decisions for patients. However, this study also highlights the limitations of flow cytometry in providing false-negative results, emphasizing the importance of using it in conjunction with DAT testing to enhance the sensitivity of AIHA detection.

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