Original Article

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Serological Characterisation of Auto-antibodies in Patients with Direct Antiglobulin Test Positive Autoimmune Haemolytic Anaemia at a Tertiary Care Teaching Hospital in Tirupati, India

Transfusion Medicine Section

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ABSTRACT

Introduction: Haemolysis in Autoimmune Haemolytic Anaemia (AIHA) is a result of Immunoglobulin G (IgG) or Immunoglobulin M (IgM) auto-antibodies with or without complement components binding to the Red Blood Cell (RBC) surface and initiating its destruction. Serologic evidence is provided by autocontrol or Direct Antiglobulin Test (DAT). Diagnostic work-up is essential as the management depends on the antibody type. Characteristics of the bound antibody and the target antigen determine the degree of haemolysis. Serological characterisation in AIHA helps to differentiate into its various types which help the clinician to decide on the treatment to be given.

Aim: To serologically characterise the auto-antibodies in patients with DAT positive AIHA at a tertiary care teaching hospital.

Materials and Methods: This cross-sectional study was carried out in the Department of Transfusion Medicine, Sri Venkateswara Institute of Medical Sciences, Tirupati, Andhra Pradesh, India, from March 2019 to February 2020. A 40 consecutive patient samples were included in the study. Characterisation of antibody was done using polyspecific Anti-Human Globulin (AHG) reagent followed by mono-specific AHG reagent by gel method. If antibody was of IgG type, then the subclass was determined by a mono specific anti-IgG1 and anti-IgG3 gel card. Association between antibody types, subtype, and strength of DAT with severity of haemolysis were compared using Chi-square/Fisher's-exact test. A p-value of less than 0.05 was considered statistically significant.

Results: The total study population was 40 patients. The mean age of the study population was 45 years (range 13-78). Out of 40 patients, males were 30 (75%) and females were 10 (25%). The primary and secondary causes for AIHA include 4 (10%) and 36 (90%) respectively. Among 40 patients, 22 (55%) patients had IgG antibody alone, 17 (42.5%) patients had IgG antibody with combination of other antibodies and 1 (2.5%) had only complement (C3d). IgG1 was identified in 7 (18%) of patients, combination of IgG1 and IgG3 in 3 (7.7%). There was a significant association with IgG+combination (p-value=0.03), IgG1+IgG3 (p-value=0.029) and strength of reaction (p-value=0.003) with respect to severity of haemolysis.

Conclusion: Presence of multiple antibodies, presence of IgG1 and IgG3 and with complement combination and presence of higher grading of reaction in gel column were associated with severity of haemolysis. We recommend that serological characterisation of auto-antibody in AIHA would help the clinician in assessing the severity of haemolysis so that management can be done appropriately.

Keywords: Autoimmune diseases, Haemolysis, Immunoglobulin, Red blood cell

INTRODUCTION

The AIHA is characterised by accelerated red cell destruction with shortened red cell survival due to auto-antibodies directed against patient's own red cells [1]. Symptoms of AIHA can vary from mild anaemia to life threatening complications secondary to severe anaemia. The incidence of AIHA in adults is one to three cases per 100,000 per year [2]. The disease peak incidence is between sixth and seventh decade and the frequency is more in females when compared to males [3].

The criteria requires to diagnose AIHA are serologic evidence of an auto antibody and clinical or laboratory evidence of haemolysis. Haemolysis in AIHA is a result of Immunoglobulin G (IgG) or Immunoglobulin M (IgM) auto-antibodies with or without complement components binding to the RBC surface and initiating RBC destruction. Serologic evidence is provided by autocontrol or DAT [1].

A positive DAT does not conclusively distinguish auto-antibodies of clinical importance from those without. Characteristics of the bound antibody and the target antigen determine the degree of haemolysis. Serological characterisation in AIHA helps to differentiate auto-antibodies into its various types. It informs the clinician regarding the disease course and to decide on the treatment to be given.

Hence, with this background, as there were only limited studies available from our country [4], authors performed to serologically characterise the auto-antibodies in patients with DAT positive AIHA at a tertiary care teaching hospital and to characterise its subtype, DAT strength and their association with severity of haemolysis.

MATERIALS AND METHODS

This cross-sectional study was carried out at the Department of Transfusion Medicine, Sri Venkateswara Institute of Medical Sciences, Tirupati, Andhra Pradesh, India, from March 2019 to February 2020. The study was started after obtaining approval from Institutional Ethics Committee (IEC no 891- Roc AS/11/IEC/SVIMS/2017 dated 28.03 2019).

Inclusion criteria: All the patients who were positive for poly specific DAT and those who were willing to participate in the study by giving written informed consent were included in the study.

Exclusion criteria: Patients of alloimmune haemolytic anaemia like haemolytic disease of newborn, haemolytic transfusion reaction were excluded.

Review of our Departmental data during the last two years showed, on an average, 400 samples per year were submitted for DAT, of

which, 10% of the samples were found to be positive. Hence, 40 consecutive patient samples were included in the study.

Study Procedure

The following laboratory investigations like complete haemogram, percentage of reticulocytes, total serum bilirubin, and serum Lactate Dehydogenase (LDH) was done. The laboratory parameters used to categorise severity of haemolysis are total serum bilirubin (>2 mg/dL), haemoglobin (<9 g/dL), percentage of reticulocyte (>2%), serum LDH (>500 IU/mL) [5,6]. Haemolysis was classified into severe if all the above parameters were fulfilled, or classified into moderate if any of the two or three above parameters were abnormal as per the criteria reported in a study by Das SS et al., [4]. A score of 2 was given if any two of the above parameters were present and a score of 3 was given if three parameters were present.

Two millilitre of ethylene diamine tetraacetic acid blood sample from each patient was collected. Column agglutination technology by gel card method was used to serologically characterise the auto-antibodies. Characterisation of antibody was done using polyspecific Anti-Human Globulin (AHG) reagent containing Anti-IgG+C3d (BioRad, Switzerland) initially as per our Departmental standard operating procedure.

If sample showed positive DAT with polyspecific AHG, it was further tested as per the manufacturer's instructions (ID-Card DC Screening I, BioRad, Switzerland) with mono specific AHG which contains antibody to IgG, IgM, IgA, and complement factors like C3c and C3d. Positive DAT reactions were graded as 1+, 2+, 3+ and 4+ as per the manufacturer's instructions.

If antibody was of IgG type, then the subclass was determined by a mono specific anti-IgG1 and anti-IgG3 gel card (ID-Card DAT IgG1/IgG3, BioRad, Switzerland) as per the manufacturer's instructions. This card consist of monoclonal anti-IgG1 in two different dilutions (1:1 and 1:100), anti-IgG3 in two different dilutions (1:1 and 1:100), anti- IgG (rabbit) in 1:10 dilutions and a negative control.

STATISTICAL ANALYSIS

Data was entered in Microsoft Office Excel (Microsoft Corporation, Redmond, WA). All continuous data was expressed as mean, standard deviation and median (Inter quartile range) and were compared using unpaired student t-test/Mann-Whitney U test as appropriate. Categorical variables were expressed as percentages and were compared using chi-square/Fisher's-exact test as appropriate. Association between antibody types, subtype, and strength of DAT with severity of haemolysis were compared using chi-square/Fisher's-exact test as appropriate. Multivariate logistic regression analysis was done for parameters showing statistical significance. A p-value of less than 0.05 was considered statistically significant. The data was analysed with Statistical Package for the Social Sciences (SPSS) version 21.0 (SPSS, Inc., Chicago, IL).

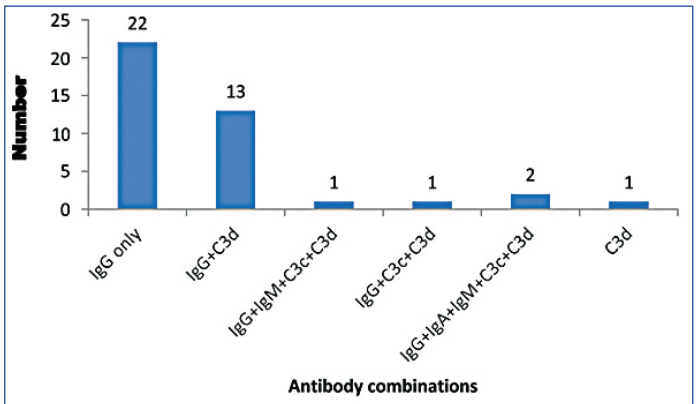
RESULTS

The total study population was 40 patients. The mean age of the study population was 45 years (range 13-78). Out of 40 patients, males were 30 (75%) and females were 10 (25%). The primary and secondary causes for AIHA include 4 (10%) and 36 (90%) respectively. Autoimmune diseases like Systemic Lupus Erythematosus (SLE), rheumatoid arthritis (38.9%) were the most common cause of secondary AIHA. Other causes include lymphoproliferative disorder, malignancy and infections. There was no significant association between the causes of AIHA (primary and secondary) and severity of haemolysis (p-value=0.54) by Chi-square test.

None of the subject fit into severe haemolysis criteria and all the 40 study population fit into moderate criteria with different scores i.e., score 2 (with any of the two laboratory parameters) and score 3 (with any of the three parameters). Eighteen (45%) subjects were found to had moderate haemolysis with score 3 and 22 (55%) with score 2. Analysis of data was done between score 2 and score 3 as there was no patients with severe haemolysis criteria.

The mean value of haemoglobin was 6.77g/dL (SD 2.39), median value of serum LDH was 511IU/dL (IQR-250,552) and that of serum bilirubin was 2.05 mg/dL (IQR-0.7,2.7) and that of reticulocyte was 1.75% (IQR-0.6,2.5). There was a statistically significant difference in median values of serum bilirubin (p-value=0.026), percentage of reticulocyte count (p-value=0.001) with score 2 and 3 by Mann-Whitney U test. Whereas, there was no significant difference between mean values of haemoglobin (p-value=0.22), median values of LDH (p=0.714) with score 2 and 3 by independent t-test and Mann-Whitney U test respectively.

Among 40 patients, 22 (55%) patients had IgG antibody alone, 17 (42.5%) patients had IgG antibody with combination of other antibodies and 1 (2.5%) patient had only C3d [Table/Fig-1]. Among 39 IgG patients, IgG1 was identified in 18% of patients, combination of IgG1 and IgG3 in 7.7% and 74.3% had neither IgG1nor IgG3. Among 22 IgG alone patients, 2 had IgG1 antibody component, 1 had IgG1+IgG3 and 19 had neither IgG1 nor IgG3 subtype. Among 17 IgG plus other antibody combination like IgM, C3d etc., 5 had IgG1subtype, 2 had IgG1+IgG3 subtype and 10 had neither IgG1 nor IgG3 subtype.



[Table/Fig-1]: Various combinations of auto-antibodies in patients.

Moderate haemolysis with score 2 was seen in 13 patients with IgG antibody alone and in 9 patients with IgG and combination of other antibodies. In patients with moderate haemolysis with score 3, 9 patients had IgG antibody alone and 9 patients had IgG with combination of other antibodies [Table/Fig-2]. There is a significant association between presence of combination of antibodies (IgG+ combination) and severity of haemolysis when compared to IgG alone (p-value=0.043).

Antibody type	Score 2 Number (%)	Score 3 Number (%)	Odds ratio (OR)	95%CI	p-value
IgG	13 (59.1)	9 (50)			
IgG+ combination	8 (36.4)	9 (50)	1.44	1.32-5.06	0.043
C3d alone	1 (4.5)	-			
Total	22 (55)	18 (45)			

[Table/Fig-2]: Association of antibody type with severity of haemolysis by Fischer-exact test.

Out of 7 IgG1 alone patients, 4 had score 2 and 3 patients had score 3. IgG1+IgG3 constitute a total of 3 patients out of which 1 had score 2 and 2 had score 3. Neither IgG1 nor IgG3 constitutes a total of 30 patients out of which 16 had score 2 and 13 had score 3 [Table/Fig-3]. There was a significant association between IgG1 and/or IgG3 and severity of haemolysis when compared with other combinations (p-value=0.017) but there was no significant association when there was absence of IgG1 and IgG3 (p-value=0.433).

IgG1 and/or IgG3 without complement was seen in 3 patients with score 2 and none with score 3. IgG1 and/or IgG3 with complement

IgG subtype	Score 2 Number (%)	Score 3 Number (%)	OR	95%CI	p-value
IgG1alone	4 (19)	3 (16.7)			
IgG1 + IgG3	1 (4.8)	2 (11.1)	1.65	1.37-5.68	0.017
Neither IgG1 nor IgG3	16 (76.2)	13 (72.2)	0.38	0.03-4.58	0.433
Total	21 (53.85)	18 (46.15)			

[Table/Fig-3]: Association of IgG subtypes with severity of haemolysis by Fischer-exact test.

was seen in 2 patients with score 2 and 5 with score 3. Neither IgG1 nor IgG3 without complement was seen in 10 patients with score 2 and 9 with score 3. Neither IgG1 nor IgG3 with complement was seen in 6 patients with score 2 and 4 with score 3 [Table/Fig-4]. There was a significant association between severity of haemolysis and IgG1 and/or IgG3 with complement combination (p-value=0.019) and there is no significant association between severity of haemolysis when there was absence of IgG1 and/or IgG3 with or without complement (p-value=0.750).

Type of antibody Number (%)	Score 2 Number (%)	Score 3 Number (%)	OR	95%CI	p-value
IgG1 and/or IgG3 without complement	3 (14.3)	0 (0)			
IgG1 and/or IgG3 with complement	2 (9.5)	5 (27.8)	2.13	3.15-9.45	0.019
Neither IgG1 nor G3 without complement	10 (47.6)	9 (50)			
Neither IgG1 nor G3	6 (28.6)	4 (22.2)	1.44	0.41-5.06	0.750

[Table/Fig-4]: Association of IgG subtypes and complement with severity of haemolysis with complement by Fischer-exact test.

The strength of the DAT 4+ was seen in 15 patients with score 2 and 10 with score 3. The strength of the DAT 3+ was seen in 4 patients with score 2 and 2 with score 3. The strength of the DAT 2+ was seen in 2 patients with score 2 and 4 with score 3. The strength of the DAT 1+ was seen in 1 patients with score 2 and 2 with score 3. Patients with 4+ reactions are found to had significant association with severity of haemolysis when compared to lesser grade of reaction (p=0.007).

The antibody subtype among IgG1 in 1:1 titre was seen in 3 patients in score 2 and 3 in score 3. The antibody subtype among IgG1 in 1:100 titre was seen in 1 patients in score 2 and none in score 3. The antibody subtype among combination of IgG1 in 1:1 and IgG3 1:1 titre was seen in 1 patients in score 2 and none in score 3. The antibody subtype among IgG1 in 1:100 titre and IgG3 in 1:100 was seen in none patients in score 2 and 2 patients in score 3. There was no significant association between severity of haemolysis and IgG1titres (p-value=0.367) and with IgG3 combination titres (p-value=0.489).

Regression analysis for those variables which had a p-value of <0.05 was done and it showed a significant association with IgG+combination (OR 1.32, 95% CI 1.43-4.89, p-value=0.03), IgG1+IgG3 (OR 1.78, 95% CI 2.68-8.54 p-value=0.029) and 4+ strength of reaction (OR7.95, 95% CI 2.45-12.01, p-value=0.003) with respect to severity of haemolysis.

DISCUSSION

The serologic characterisation helps to determine whether the haemolysis has an immune basis and if so, what type of immune haemolytic anaemia is present. This is important because the treatment for each type is different. Hence, it is important to serologically characterise auto-antibodies in AIHA to effectively predict the prognosis and disease outcome.

In our study population, the male to female ratio was 3:1 with the mean age group of 45 years (range 13-78). More number of cases was seen in the fourth decade which was similar to another study where they had reported high incidence of AIHA above 40 years age [7]. One Indian study reported that the median age of their study population was 37 years with male to female ratio of 5:8 [8]. The high male to female ratio in our study might be because of our small study population. Another reason could be, in country like India, females hesitate to come to hospitals and usually take their own home remedies due to financial and social constraints making male preponderance in our study.

In our study, 4 (10%) patients were diagnosed to have primary AIHA and 36 (90%) patients had secondary AIHA. Though secondary AIHA is comparatively more which is similar to other studies, the incidence of secondary AIHA is very high when compared to other studies. A study from Lucknow reported 38.5% and 61.5% of primary and secondary AIHA respectively [4] whereas another study reported 55% and 45% respectively [9]. The higher incidence of secondary AIHA in our study might be due to complications of underlying disorder making the patient to attend the hospital. The most common secondary cause of AIHA in our study was autoimmune diseases (38.8%) which are similar to various other studies [4,10]. Some studies observed lympho-proliferative disorders as the leading causes [11,12].

Haemolysis was classified into severe and moderate as per the criteria laid down in one study [4]. But, in our study no severe haemolysis was noted and all 40 study population had moderate haemolysis. Of the total 40 patients, 22 (55%) patients were categorised to had moderate haemolysis with score 2 and 18 (45%) with score 3. There was no significant association between cause of AIHA and severity of haemolysis (p-value=0.54) which might be due to lesser sample size in one of the arm.

In our study, there was a statistically significant difference in median values of serum bilirubin (p-value=0.026), percentage of reticulocyte count (p-value=0.001) with score 2 and 3 whereas there was no significant difference between mean values of haemoglobin (p-value=0.22), median values of LDH (p-value=0.714) with score 2 and 3. However, one study observed a significant association between the above said laboratory parameters and the severity of in vivo haemolysis [6]. Another study reported no correlation between the haemoglobin level and severity of haemolysis [10].

Majority of our patients were positive for IgG auto-antibody either alone or in combination. Out of 40 patients, 39 (97.5%) were found to be positive for IgG and only one patient (2.5%) was positive for C3d alone. Out of 39, 22 (56.4%) patients were positive for IgG auto-antibody alone whereas 17 (43.6%) patients were found to be positive for IgG along with other auto-antibodies. This is similar to the finding noted in one Indian study, where 68.5% of their patients had solitary IgG [4]. In another study, almost all the patients had IgG auto-antibodies [3]. We found a significant association between presence of multiple antibodies and severity of haemolysis when compared to IgG alone (p-value=0.04).

The IgG1 was identified in 18% of patients, as compared to 7.7% of patients, who were identified to had combination of IgG1 and IgG3 and rest 74.3% had neither IgG1 nor IgG3. This is in contrast to one study where IgG1 was detected in 53.8%, IgG1 and IgG3 combination in 34.6% and IgG3 alone in 7.7% [13]. Two different studies reported neither IgG1 nor IgG3 in 51.2% and 3.8%, respectively [4,13]. There was a significant association between IgG1 and/or IgG3 and severity of haemolysis (p-value=0.017) but there was no significant association when there was absence of IgG1 and IgG3. It revealed that patients who had IgG1 and IgG3 were 1.65 times (95% CI-1.37, 5.68) more likely to present with severity in haemolysis as compared to patients without IgG1 or IgG3. The present study findings were concordant to one study, where the patients had severe haemolysis when their red cells were coated with IgG1 and/or IgG3 [14]. We did not find significant association between severity of haemolysis and IgG titres (p-value=0.367) which might be due to lesser sample size.

Complement was found in 45% of our study population (43.5% in combination with other antibodies and 2.5% as alone). One study documented that 72% of patients had complement [15]. In our study, there was a significant association between severity of haemolysis and IgG1 and/or IgG3 with complement combination (p-value=0.019) and there was no significant association when there was absence of IgG1 and/or IgG3 with or without complement (p-value=0.750).

The present study revealed that patients who had 4+ reaction were 7.32 times (95% CI 2.35, 12.65) more likely to present with severity in haemolysis when compared to patients with lesser grading of reaction. Gopal KR et al., showed a strong correlation between the strength of DAT and severe haemolysis [16]. A similar study by Das SS et al., reported that greater strength of DAT was associated with increased severity of haemolysis [4]. However, a study done from New Delhi did not find any correlation between them [17].

Limitation(s)

Limitations of the present study include lesser sample size as the number of patients registered during the study period was less. Absorption studies were not done to assess the presence of alloantibody in addition to auto-antibody which would have influenced on the severity of haemolysis.

CONCLUSION(S)

Hence, authors concluded that this AIHA study population had predominantly IgG antibody followed by IgG and complement combination and occasional cases of complement alone and IgM and IgA. IgG1 was the most common subtype with majority having a titre of 1:1. We found that presence of multiple antibodies, presence of IgG1 and IgG3 and with complement combination and presence of higher grading of reaction in gel column, laboratory evidence of increased serum bilirubin and reticulocyte count were associated with severity of haemolysis.

It is recommended that serological characterisation of auto-antibody in AIHA would help the clinician in assessing the severity of haemolysis so that management can be done appropriately. However, further studies with more sample size are required to assess the significance of various other factors.

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