ORIGINAL ARTICLE

Determination of Weak "D" Antigen among Rhesus Negative Pakistani Blood Donors

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ABSTRACT

Objective: Determination of weak "D" antigen, to highlight the importance of "Du Testing" among the Rhesus-negative blood donors.
Study Design: Cross-sectional study.
Place and Duration: Study was conducted at department of Hematology, Allama Iqbal Medical College (AIMC), Lahore, during the period of January 2013 and December 2015.
Materials & Methods: Five different tertiary care and four secondary care hospitals of Lahore and six different institutes/universities including individuals visiting to blood banks for determination of their blood groups were included in study. Two ml of blood sample was collected in EDTA-containing vial and analyzed for determination of ABO/Rhesus (Rh) typing; all Rh-negative samples were further processed for the Du Testing according to standard protocol and commercially available monoclonal antibody.
Results: Out of total 55,874 participants only 8.0% (n = 4,454) were Rh-negative. Among these Rh-negative samples, 99.0% (n = 4,410) samples were negative even after "Du Testing". Only 0.98% (n = 44) were weak "D" positive, in Rh-negative samples.
Conclusion: every Rh-negative individual should be processed for the detection of Weak "D" antigen by "Du Testing" as it may not be detected by immediate spin tube method.
Keywords: Rh negative, weak D antigen, Du Testing.

Introduction

Until the 19th century the blood transfusion procedure was unsafe, but this mystery was solved in 29th century with the discovery of ABO and Rh blood group antigens.[1] The discovery of Rh system by Levine and Stetson in 1939 was great to break through in transfusion medicine. It is the most significant blood group system after ABO. Studies have shown that a significant proportion of patients who's RBCs lack "D" antigen makes anti-D, if they have exposed to the "D" antigen by blood transfusion. Therefore, determination of Rhesus (Rh) phenotype is of critical importance.[2] Anti-D of the Rh blood group system is clinically important. It causes hemolytic transfusion reactions (HTR) and hemolytic disease of newborn (HDN). In Rh blood group system there are mainly five antigens inclusive of "D", "C", "c", "E", and "e" which are clinically significant, among these "D" antigen is highly immunogenic.[3] Genetic nature of Rh system showed that it is most complex blood group in

human. It comprised of two genes *RHD* and *RHCE* found on chromosome no 1. *RHD* Gene is responsible for expression of *RHD* protein with "D" antigen, while *RHCE* gene encodes the *RHCE* protein with "Cc/Ec" antigen. Individuals having RBCs with "D" antigens are labeled as Rh-D+ve and those without "D" antigen were as Rh-D–ve.[3] Different possible mechanisms for the lack of weak "D" antigen may be due to Gene deletion, Gene mutation, Gene rearrangement and Gene duplication.

The term Du antigen is used when there is a weak expression of "D" antigen on RBC. This terminology was explained by Stratton in 1946.[4] In 1984 this is further replaced by another suitable expression, the weak "D" antigen.[5] Historically RBC antigens that react with anti-D only after reacting with the indirect antiglobulin test are known as "Weak D".[6] It usually results from amino acid substitutions within the internal portion or in the membrane-crossing portion of the RhD protein.

The number of "D" antigen sites on the Rh-D+ve RBC is normally in the range of 9900 to 33000 ([7]) and the weak "D" phenotype seemed to be a quantitative disparity in the number of "D" antigen sites on red blood cell.[7]

As a result weak or no agglutination reaction is demonstrated with the anti-D in the immediate spin phase technique. With the use of flow cytometry, it was established that weak "D" subjects had at least 10 times lower expression than showed by the "D" positive subjects. [8]

The anti "D" antisera are commonly used for the detection of Rh-D antigens on RBC**.** Weak "D" individuals (who are actually Rh-positive) were commonly mistyped as Rh-negative through the use of less sensitive polyclonal antisera (anti-D) via direct agglutination testing with grave consequences. However, with the advent of highly sensitive monoclonal anti-D reagents, most weak "D" individuals are now typed as Rh-positive by indirect antiglobulin test. However, In developing countries like Pakistan where polyclonal antisera are still used in labs, mostly at the district level. Also alarming evidence exists about some immunogenic weak "D" antigens containing specimens that would not be detected by polyclonal anti-D antisera. Therefore indirect antiglobulin test may be performed to detect weak "D" in individuals who are initially typed as Rh-negative.

The diversity of the "D" antigen has opened a new debate about the interpretation of laboratory test results for weak "D" antigen and selection of blood or blood products for transfusion to avoid the risk of alloimmunization.

Materials and Methods

Ethical approval: Study protocol was approved by ethical review board of AIMC.

Study setting & duration: Department of Hematology, Allama Iqbal Medical College (AIMC), Lahore, during the period of January 2013 and December 2015.

Specimen collection: Total 55,874 participants were enrolled from five different tertiary care hospitals of Lahore (Punjab Institute of cardiology, Lahore General Hospital, Mayo Hospital, Lady Willingdon Hospital and Sir Ganga Ram Hospital) and four secondary care hospitals of Lahore (Nawaz Sharif Yakki Gate Hospital, Kot Khawaja Saeed Hospital, Mian Munshi Hospital and Said Mitha Hospital) and six different institutes/universities (University of Lahore, Lahore, Imperial college of Business Lahore, Government College University Faisalabad, Riffah International University Lahore, Hajveri University Lahore and University of Agriculture Faisalabad) through non-probability consecutive sampling technique. Individuals who were visiting to blood banks of above-mentioned settings for determination of blood groups were also included in the study. From each individual two ml blood sample was collected in the EDTA vial using standard venipuncture technique.

Sample processing: All specimens were processed for ABO and Rh typing. For determination of ABO typing, 5% red blood cell suspension was mixed with anti-A, and anti-B sera in test tube at room temperature and examined for agglutination macroscopically and microscopically after centrifugation. Immediate spin tube method was used for the determination of Rh typing. All individual's 5% red blood cell suspension was mixed with Anti-D sera in test tube and examined for agglutination macroscopically and microscopically after centrifugation. Proper Agglutination was considered a positive reaction and typed as Rh-positive.

Du testing: All specimens found negative for agglutination were typed as Rh-negative and further processed for detection of weak "D" antigen by Du Testing. An equal volume of 5% washed RBC and Anti-D reagent were mixed and incubated at 37°C for 30 minutes. After centrifugation the cell button was re-suspended and agglutination was observed macroscopically and microscopically. Reactions which showed agglutination were indicated Rh-positive. In case there was no agglutination the mixture was washed 3 times with normal saline. After the last wash, saline was decanted off and two drops Anti-Human Globulin (AHG)

were added. After centrifugation, Macroscopic and microscopic agglutination was observed and any agglutination at this stage was recorded as weak "D" positive reaction. The positive control is made of check cells (washed "O" positive red blood cells with anti-D) and washed "O" positive cells with 0.9% Normal Saline was used as negative control. All negative results were observed for agglutination after addition of check cells.

Data presentation & statistical analysis: Data was entered into Statistical Package for Social Sciences SPSS version 17). After entering the data it was analyzed descriptively and analytically.

Results

Out of total 55,874 participant male gender was dominant (55%) than females (45%) [**Figure-1].**

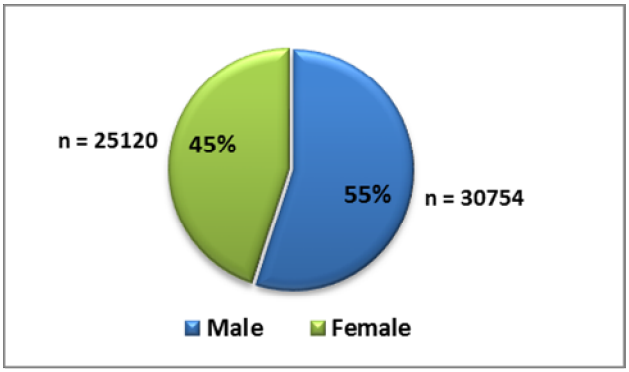

Figure-1. Gender distribution

The overall frequency of ABO blood groups in male and female was calculated, showed a high frequency of blood group "B" in both genders. The frequency of Rh-positive blood groups was 84% higher than Rh negative blood groups [**Table-I].**

In male individuals frequency of ABO blood group with respect to Rh-D was calculated which showed a high frequency of "B" positive 34.9% (n = 9874) followed by "O" positive 31.8% (n = 8995), "A" positive 26.1% (n = 7397) and "AB" positive 7.0% (n = 1989). In the case of Rh factor negative male individuals B-ve showed high frequency 45.0% (n = 1125) as compare to other blood types followed by "O" negative 39.7% (n = 993) "A" negative 7.7% (n = 193) and "AB" negative 7.5% (n = 188) [**Table-II].**

Table-II: Gender Based Distribution of ABO and Rh status

ABO	Gender	Rh antigen		Total	chi-square P value
		Positive	Negative		
A	Male	7397 (26.1%)	193 (7.7%)	7590 (24.6%)	X2= 965.834 P = 0.000
	Females	4383 (19.9%)	997 (50.9%)	5380 (21.4%)	
B	Male	9874 (34.9%)	1125 (45.0%)	10999 (35.7%)	X2= 672.826 P = 0.000
	Females	9021 (38.9%)	165 (8.4%)	9186 (36.5%)	
O	Male	8995 (31.8%)	993 (39.7%)	9988 (31.2%)	X2= 2.029 P = 0.154
	Females	5724 (24.7%)	681 (34.8%)	6405 (25.4%)	
AB	Male	1989 (7.0%)	188 (7.5%)	2177 (7.0%)	X2= 121.769 P = 0.000
	Females	4037 (17.4%)	112 (5.7%)	4149 (16.5%)	
Total	**Male**	**28255 (91.8%)**	**2499 (8.1%)**	**30754 100.0%)**	
	Females	**23165 (92.2%)**	**1955 (7.7%)**	**25120 (100.0%)**	

In female participants frequency of ABO blood group with respect to Rh factor showed a high frequency of "B" positive 38.9% (n = 9021), followed by "O" positive 24.7% (n = 5724), "A" positive 19.9% (n = 4383), and "AB" positive was 17.4% (n = 4037). In the case of Rh-negative female individuals A-ve was 50.9% (n = 997) which was the highest frequency as compared to another blood type as "O" negative was 34.8% (n = 681), "B" negative 8.4% (n = 165), and "AB" negative was 5.7% (n = 112) [**Table-II].**

In our study the status of Weak "D" antigen in Rh-negative subjects was also calculated, which showed that out of total 8.0% (n = 4454) Rh-D negative specimens, only 0.98% (n = 44) were positive for weak "D" antigen and remaining 99.0% (n = 4410) were weak "D" negative

Table-I: Rh Factor Based Frequency of ABO Blood Groups (n=55874)

Blood Groups	Gender			Rh antigen		Chi-square P value
	Male Donors	Female Donors	Total	Positive	Negative	
A	7590 (24.7%)	5380 (21.4%)	12970 (23.2%)	11780 (22.9%)	1190 (26.7%)	
B	10999 (35.8%)	9186 (36.5%)	20185 (36.1%)	18895 (36.7%)	1290 (28.9%)	
O	9988 (32.5%)	6405 (25.4%)	16393 (29.3%)	14719 (28.6%)	1674 (37.5%)	$X^2 = 708.268$ **P = 0.000**
AB	2177 (7.0%)	4149 (16.5%)	6326 (11.3%)	6026 (11.7%)	300 (6.7%)	
Total	**30754 (100.0%)**	**25120 (100.0%)**	**55874 (100.0%)**	**51420 (92.0%)**	**4454 (8%)**	

[**Table-III**].

Table-III: weak "D" positivity among Rh-negative participants n=4454

Gender	Weak-D Antigen		Total Rh Negative	Chi square P value
	Positive	Negative		
Male	29 (1.1%)	2470 (98.8%)	2499 (56.1%)	$X^2 = 1.734$ P = 0.188
Female	15 (0.7%)	1940 (99.2%)	1955 (43.8%)	
Total	**44 (0.98%)**	**4410 (99.0%)**	**4454 (100%)**	

In our study of 55,874 blood donors, we observed that 92.0% (n = 51,420) and 8.0% (n = 4,454) were Rh+ve and Rh–ve respectively [**Table-I**].

Discussion

In the field of transfusion medicine, the discovery of blood group antigens and their role marked the revolutionizing step in the development and success of blood group systems. Appropriate assignment of Rh status especially Rh-D is of critical importance. We investigated the Rh-D status of blood donors in the Pakistani population, which has remained completely unexplored so far. In Asian countries a wide range of variants of Rh-D is reported in the literature.

From Pakistan a study conducted by Usman et al 2013, reported 93% Rh-D positive and 7% Rh-D negative, and 0.8% weak "D" positive.[9] A similar study was conducted in India by Nitin Agarwal et al 2013, reported 94.8% RhD-positive, 5.2% RhD-negative. And only 0.09% of Rh-negative and 0.005% of the total population turned out to be positive i.e. weak D (10)**.** Seema acharya et al 2011, from India reported 12.62% RhD-negative 0.135% weak "D" positive.[5] Another study at Uttara khand from India, reported 94.8% Rh-positive, 5.2% Rh-negative. The frequency of weak D was 0.09%. Even less than that which is found in our study (0.98%). Makro et al 2010, reported 7.19% of the donors Rh-negative and the weak "D" variant 0.01% in Indian population.[3] A study conducted in Africa in 2008 by Okrah reported 92.25% RhD-positive and 7.75% RhD-negative and 6.45% weak "D" positive blood donors (11). In another study Xhetani et al 2014, reported 10.86% RhD-negative donors, out of which 0.14% were weak "D" in Albanian population.[12]

There are three genetic mechanisms for the acquisition of weak expression of the D antigen. The first mechanism reveals Person may be inheriting the RHD gene which codes for a weakly expressed "D" antigen. Secondly "D" antigen may be weakly expressed due to the presence of "C" antigen in the Transposition on the conflicting Chromosomes such as Dce/dCe genotype.[5] Thirdly Partial "D" antigen when one or more Epitopes of the "D" antigen are absent.

Different incidence rates of Rh-negative and weak "D" have been reported around the globe which is in the range of 3-25% and 0.2% -1% respectively.[5] This rate is mainly influenced by Genetic diversity among different study populations. In Indians Rh-negativity was reported approximately 5%, while the prevalence of weak "D" antigen is 0.3 - 0.5%. Though the incidence varies from community to community.[5, 10] Different studies have reported the prevalence of weak "D" from 05 to 10% in different countries.[11, 13] The minimum prevalence was reported from Caucasian by Flegel which is about 0.4%.[13] The incident rate of weak "D" antigen in Pakistani population was reported as 0.8% .[10]

There is a misconception that a person with weak "D" phenotype could not develop anti-D in comparison to Partial "D" due to a low number of complete "D" antigens. Different studies and Literature have shown that Partial "D" which is due to altered genes [14, 15] can cause anti-D alloimmunization.[16, 17]

Because RhD antigen is highly immunogenic, when the RhD-negative recipient is transfused blood by the RhD-positive donor, then the recipient will develop Anti-D alloantibodies. Another important fact is the development of hemolytic diseases of newborn (HDN), when sensitized Rh-negative women conceive a Rh-positive fetus, due to the exposure of anti-D antibodies across the placenta to the fetus.[10]

Hemolytic transfusion reaction would occur when a weak "D" antigen is transfused to the sensitized RhD-negative recipient. But transfusion of weak "D" RBCs to Rh-negative subjects would result in the development of alloimmunization only. Several evidence has been reported the favoring development of alloimmunization, due to Weak "D" and Partial "D" antigen. [14, 18]

Now with the approval of World Health Organization (WHO), American Association of Blood Banks (AABB), German and British committee for standards in hematology/Blood Transfusion, it has become compulsory to determine the status of Weak "D"/ Partial "D" among blood donors and recipient as to safely declare RhD-negative especially when polyclonal Anti-D reagents are used for Rh typing.[15] Similar recommendations were given by the Society of Obstetricians and Gynecologists of Canada for weak "D" testing in pregnant women. [16]

The efficacy and accuracy of AHG test for detection of weak "D" antigen are reported as well accepted in literature.[19] However, the utilization of different Anti-D reagents in the market is controversial. It is necessary that all blood banking centers should follow instructions of the particular country for blood typing and selection of Anti-D Regents.

However, still after many years of the discovery of the weak "D" antigen, its clinical significance, immunogenicity and guidelines are debated. Therefore the blood banks investigate all weak "D" negative individuals for weak "D" antigen by AHG test. Although the complete answer to solving divergence of weak "D" and "D" variants, can only be done by novel techniques like Ultra Violet (UV) Spectrophotometry and molecular analysis.[10] In developing countries the use of anti-human gamma globulin test for weak "D" determination has still worth especially for pregnant women and donors in rural areas and district levels. Therefore, issues regarding weak "D" phenotype should be undertaken in combination with molecular studies to formulate beneficial, cost effective and standardized guidelines.

Conclusion

Detection of weak "D" antigen must be an essential part of blood grouping and compatibility testing for safe blood transfusions. All Rh-negative individuals should be processed for Weak "D" antigen by "Du Testing" as it may not be detected by immediate spin tube method.

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