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Prevalence of weak D among the patients and donors attending in a tertiary hospital Bangladesh.

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Abstract:
Background:
Rhesus (Rh) blood group with variable expression of D antigen is one of the complex systems in immunohematology. Weak D antigen is a phenotype where the D antigen is weakly expressed on red blood cells, and this antigen cannot be detected by routine methods. Therefore, Weak D RBCs demonstrate reduced quantities of the D antigen. As a result, weak or no agglutination reaction is demonstrated by these RBCs with the anti D reagents at the immediate spin phase. About 0.1 to 2 percent of white Caucasians have this Rh phenotype. Missense mutations observed in the alleles of all weak D types have been demonstrated to be the probable cause for the reduced antigen D expression in these cases[10]
Aims:
This study was conducted to determine the frequency of Rh D negativity and weak D antigen among healthy blood donors andpatients to review the clinical significance of weak D antigen pertaining to Rh D-negative transfusions.
Materials and methods:
It's a cross sectional observational study based on the availability of hospital records done from January 2019 to December 2020. Samples were collected from the donors and also from the patients Their D typing was confirmed by at first by the polyspecific(IgG +IgM) commercial reagents using Tulip, Lorne ,Biorex and Immucor. The availability of using albumin, papain and anti-human globulin technique by indirect Coombs test method.
Result:
Total blood group were performed from January 2019 to December 2020 (19130+ 35920) =55050. Among them D positive were 53698 and D negative were 1352. Blood group confirmation was done for 540 donors and patients to observe discrepancy in ABO and D typing. Total 244 cases were carried out for genotype and phenotype. Among them weaker D reaction found in 7 cases. Therefore, the frequency was observed about 0.013 %. The phenotype was found R_1r in 5 cases, 1 was $r^{/}r$ and 1 was $r^{//}r$. Amongst these cases, five were pregnant woman having detected negative blood group in more than three centers, one woman of 78 years was detected Rh negative but her actual typing was weak Rh D and had history of taking Rh D positive blood therefore later she was alloimmunized with C and E, and both DCT and ICT was positive. One was a donor known Rh D negative detected in a periphery but later he was typed as weakened D. Hence donated 8 times as Rh D negative donor in different blood donation centers and in periphery.
Conclusion: *Although the frequency of weak D antigen is low (01.51%), the strong immunogenicity of Rh D antigen discarnate the need for appropriate testing for weak D antigen. This is of particular concern in Rh D-negative pregnant females as it can produce alloimmunization if accidentally given weak D antigen positive blood.*

Keywords: *Alloimmunization, Rhesus D negative, Transfusion, Weak D antigen*

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I. Introduction:

Rhesus (Rh) blood antigen were first described by Levine and Stetson in 1939 who described a patient having an antibody that agglutinated red blood cells (RBC) of 85% of ABO-compatible donors. This was a second major discovery in immunohematology after the discovery of ABO blood groups by Landsteiner in 1990. Human blood groups were divided into two major groups depending on the presence (Rh positive) or absence (Rh negative) of Rh antigen. Later, Fisher and Race published their work on Rh antigen, whereby the nomenclature of CDE was accepted. The Rh system consists of over 50 antigens, with D being the major antigen expressed by Rh D protein. Out of Rh system, 5 (D, C, c, E, and e) are important for causing clinical

complications. Molecular genetics has shown that there are two Rh genes, one encoding D, the other encoding the Cc and Ee antigens [1].

The real importance of the Rh factor was only realized when it was shown by Wiener and Peter(1940) that the sera of some people, who had experienced unexplained incompatible transfusion reactions even when given blood of the correct ABO group, contained antibodies which parallel reactions to the animal anti-Rh sera. This meant that Rh factor was antigenic in man and that the anti-Rh iso -agglutinins which were formed as a result of immunization.

Since its discovery the Rh system has been found to be composed of a series of antigens each with its corresponding antibody, so that it is much more complicated than it was first realized. However, the original Rh antigen (now we are explained d or Rh_0) and its corresponding antibody, anti Rh (anti -D) account for over 90 percent of all reactions caused by the Rh blood groups.

The standard reagent belongs to two distinct types called for convenience saline anti D or albumin anti D. This is because human anti D antibodies occur either as agglutinins or complete antibodies which can agglutinate in a saline medium cell containing the corresponding antigen or as incomplete antibodies, which may sensitize the cells in saline but only cause them to agglutinate in high protein medium. The protein medium usually employed in rh testing is bovine albumin. This may be obtained from the manufacturers as a 30% solution specially prepared for the use in Rh testing.

For further steps whatever technique is used controls are necessary. For any anti -D testing three controls should be used:

1) Known Rh positive cells with the standard anti D serum.
2) Known Rh negative cells with the standard anti D serum

D typing is always performed with two or more anti-D sera. A negative result with both reagent will mean that the blood sample under test is probably d negative-it should not, however, be regarded as completely Rh negative without further testing for the other Rh antigens. This further testing can be omitted in an emergency since a patient who is negative with two or more anti D sera or reagents. Some authors also described as high grade and low grade D^u. High grade may red cells may also be positive with both anti D sera, but such cells are treated as doubtful reactions like +, +-(w) or mixed field. Then one or both anti D reagents should be further retested, if possible, with a larger range of more reagents can also be used in a doubtful condition. If the doubtful cases to wash the red cells with saline at 370c as this will appreciably restore their diminished agglutinability. A negative indication for the testing purpose with one reagent and appositive with the other is also an indication for testing against a number of anti-D sera.

The subdivisions of D negative or weaker reactions individuals are comprises some genotypes like $R^/r$,$R^{//}r$,$r^/r$,$r^{//}r$,$R^{//}R^{//}$,$R^/R_y$, R_yR_y. But most of them foregoing subdivisions are still very rare and thus seldom encountered. We commonly ascended the genotype of CdE/cde, cde/cde ,cdE/cde,Cde/cde, that is found to be frequent in comparison to our study.

It can be seen that all but true Rh negatives(cde/cde) have either C or E or both of these antigens and if they are used as Rh negative donors for transfusion they may stimulate the production of anti C or anti E in the recipient. This means that's every donor who is negative to anti D must be tested further with anti -c or anti E. Whenever time allows it is advisable to test all D negative or weaker D should be evaluated with the phenotype testing for further safety and ensuing a complete blood report. Here We are describing about two case reports where there is a confusion in declaring a blood report and having more than three reports which has to be solved clearly in regularize their treatment purpose.

Weak D represents a D phenotype where due to reduced D antigen expression on red cells, the antigen is not detected by routine techniques (spin tube method). However, the demonstration of this weakly expressed antigen can be undertaken by prolonged incubation and the use of anti-human globulin.

Years after the discovery of this weak D antigen, it has remained a topic of controversy whether to routinely test for weak D antigen or not. The clinical implications are of concern when dealing with pregnant women.

This study was conducted with the aim to determine the frequency of weak D phenotype among Rh D-negative individuals. The objective being, to highlight its clinical implications related to the risk of alloimmunization in Rh D-negative individuals and the justification for testing it.

II. Materials And Methods:

It's a cross sectional observational study based on the availability of hospital records done from January 2019 to December 2020. Samples were collected from the donors and also from the patients Their D typing was confirmed by at first by the poly specific (IgG +IgM) commercial reagents using Tulip, Lorne, Biorex and Immucor. The availability of using albumin, papain and anti-human globulin technique by indirect Coombs's test method.

Two rows of tubes are required for these tests. The setting up of this test is placed with controls.Anticoagulated blood of the Rh D-negative donors was the sample for weak D testing. A 5% RBC saline suspension was made by washing the RBCs with isotonic saline.

For each sample, a tube was taken and labeled as test sample. A saline tube acted as control. One drop of reagent containing both (IgG+IgM) and saline were added to the test tube and control tube, respectively. One drop of 5% RBC suspension was added to each tube. After mixing, incubation was done for 30 min for sensitization. After washing the sensitized cells 3–4 times with normal saline and discarding the supernatant, two drops of Anti-human serum (Coomb's serum) were added and centrifuged for 1 min. The sediment cells were gently dislodged and examined macroscopically as well as microscopically for agglutination.

Reactions with Anti D (IgG and IgM)	Positive control Placed with anti- D with Rh D positive cell	Negative control Placed with anti D with Rh d negative cell
+ (Tulip)	+++	---
+(w)Lorne	+++	---
(--)Immucor	+	---
(--)Biorex	+-	---

After incubating the patient's sample at 37^{o}C for one and half hour

(+) Tulip	++++	-----
(+-) Lorne	+++	----
(+-) Immucor	+++	-----
W (+-) Biorex	+	-----

After incubating her sample at 37oc in 30% bovine albumin and enzyme (papain) method for one and half hour

Patients red cell suspension (5%) with albumin	Positive control	Negative control
++	+++	----
++	++++	----

Rather we have taken another fresh sample to confirm her Rhesus genotype and phenotype. One tube has been taken with anticoagulant another was without anticoagulant. All tests have been done on test tube method.

III. Results:

In this study we found Rh positive were 97.54% and negative were 2.45%. Weaker D reaction found in 7 cases. Therefore, the frequency was observed about 0.013 %. The phenotype was found R1r in 3 cases, 2 was R_2r,1 was r$^{/}$r and 1 was r$^{//}$r. Among these cases, three were pregnant woman having detected negative blood group in more than three centers, one woman of 56 years was detected Rh negative but her actual typing was weak Rh D and had history of taking Rh D positive blood therefore later alloimmunized with C and E and 1 was woman of 82 years with history of taking 7 units of Rh-negative blood and DCT remains positive but ICT was negative.1 was a donor known to be detected in a periphery as Rh D negative blood. Hence donated 8 times as Rh D negative donor in different blood donation centers and in periphery.

Table 1: Total distribution of blood group:

Year	Rh D positive %	Rh D negative %
2019(January to December)	35063 (97.61%)	857(2.38%)
2020(January to December)	18635 (97.41%)	495(2.58%)

Table 2:
Distribution of D^{u} or D^{w}:

Total 7 cases	phenotype	genotype
3 were	CcDee	CDe/cde (R_1r)
two were detected as	ccDEE	cDE/cdE (R_2r)
One was detected as	CCdee	Cde/Cde (r$^{/}$r)
One was detected as	ccdEE	cdE/cdE (r$^{//}$r)

IV. Discussion:

The RH genes RHD and RHCE encode two proteins that represent the clinically most important blood group system defined by the sequences of red cell membrane proteins. RHD and RHCE, encoding the Rh proteins (D and Cc/Ee, respectively), are organised in tandem on chromosome 1p34-p36 and probably derived from duplication of a common ancestral gene. Many RH genes carry point mutations, or have rearrangements and exchanges between RHD and RHCE which result from gene conversion events. RHCE encode hybrid proteins that have RhCE-specific amino acids in RhD, or RhD-specific residues in RhCE. These might generate new antigens in the Rh blood group system, and alter or weaken expression of the conventional antigens[1,2.]

Reduced expression of D antigen occurs in an estimated 0.2%–1% of Caucasians. Historically, red blood cell antigens that react with anti-D only after extended testing with the indirect antiglobulin test are called weak D. Weak D expression primarily results from single point mutations in RHD which encode amino acid changes predicted to be intracellular or in the transmembrane regions of RhD. These affect the efficiency of insertion, and, therefore, the quantity of RhD protein in the membrane, reflected in the reduced number of D antigen sites on the red blood cells. Red blood cells with partial D antigen type as D-positive, but individuals often produce anti-D when stimulated by transfusion or pregnancy. Some partial D, similar to weak D, result from point mutations in RHD that cause single amino acid changes. But, in contrast to weak D, these changes are located on the extracellular regions and alter or create new epitopes[1,2.]

The simple division of the Rh system into Rh positive that is D positive and Rh-negative means D negative using anti –D sera only. Therefore, extended use of other sera (anti- C, anti –E etc.) are also allow in routine Rh testing. In our regular practice it can be seen that all but true Rh negatives (cde/cde), after which Cde/cde and cdE/cde are most frequent. Individuals with these subgroups are all capable of making anti D if stimulated by the D antigen obviously either due to transfusion or pregnancy.

When the normal D antigen shows weekend expression then some additional evaluation needs to be permitted. Moreover, in addition increased incubation time and also more than three different types of polyclonal reagents should be used to verify the test results.

Hence during arising any confusion of determining such steps these cells require additional steps which may be prolonged incubation with the anti D reagent or addition of ant globulin serum after incubation with anti D. Moreover, monoclonal anti-D reagents may cause direct agglutination of some D-positive cells but could have considered as weakened reaction after the use of polyclonal reagents (IgM+IgG).

In a study from Makroo *et al* showed that the incidence Rh D positive were 92.81, Rh D negative 7.19 and weak D antigen ranges between 0.2% and 1% in white Caucasians. [1] and in Indian population and found Rh negativity in 7.19% and weak D in 0.01% population.

In a study from Desai HM et al. conserve study for 5½ year, where they estimated the prevalence of weak D using serological techniques a total of 84,697 units of blood, 4541 (5.36%) donors were Rh negative and 38 (0.8% of Rh-negative donors) were weak D positive. Of 4541 Rh negatives obtained, 3506 donors were tested for weak D, 34 donors (0.9%) were detected weak D positive.In another study from Melek Yanasik et al Weak D phenotype was detected in 228(0.12 %) out of 177,554 donors.

In this study we have found Rh positive were 97.54% and negative were 2.45%. Weaker D reaction found in 7 cases. Therefore, the frequency was observed about 0.013 %.

Table3: comparison of Rh D distribution with frequency of weak D:

Study of different literature	Rh D positive%	Rh D negative%	Weak D %
Makroo et al (1, 84, 072)	92.81	7.19	0.01
Desai HM et al (84,697)	94.63	5.36	0.9
Melek Yanasik et al (177,554)	----	-----	0.12
Our study (55050)	97.54	2.45	0.013

Furthermore, in another literature Wagner T et al[3] describes as there are more than 50 types of weak D antigen. The majority (90%) of individuals with a weak D phenotype is weak D type1, these individuals do not need Rh immune globulin prophylaxis during pregnancy. The remaining 10% of individuals with a weakened expression of D express aberrant D proteins so they should receive Rh immune globulin prophylaxis during pregnancy. [11] In our study we found 7 cases of weak D. Among these cases, three were pregnant woman having detected negative blood group in more than three centers, one was woman of 86 years having history if taking Rh D positive blood, and 1 was woman of ninety years history of taking 7 units of Rh-negative blood.

In a case report from Unger PJ et al[7] shows a mixed-field agglutination in a patient with a weak D antigen presenting as a possible fetal-maternal hemorrhage. They report a D+, multi-transfused Caucasian woman with myelodysplasia who exhibited several alloantibodies. In our setting we found one patients DCT remains positive due to transfusion with negative blood but ICT was negative.

Among these seven cases one was a donor known to be detected in a periphery as Rh D negative blood. Hence donated 8 times as Rh D negative donor in different blood donation centers and later he was confirmed in our laboratory as weak D.

In another Nirav Ramesh Dava et al [6] reporting a rare case of hemolytic disease of newborn with weak D antigen in child. But in our study, we still don't get such types of cases.

Acquiescence with ethics:

The study was conducted in accordance with the Declaration of the Department of Transfusion Medicine, Bangabandhu Sheikh Mujib Medical University and was approved by the Local Ethics.

Conflicts of interest:
None declared any conflicts of interest.
Source of funding:
No source of funding for this study.

V. Conclusion:

Weaker reaction in Rh D typing therefore provides many confusions and also problems in our laboratory working. Besides, different reports from different institutions also causes mishap as well as an issue for mismatch blood transfusion. So, the bare responsibilities are to maintain the correct procedures during confusion in any D typing. Therefore, when identified as a donor or patient, individuals should be enrolled into a specific registry to make them available to donate for other after confirming the proper phenotype. Moreover, as a patient they should be D negative. Hence, in case of suspected alloimmunization like who has to be undergone multiple surgical procedures rather Hemoglobin is in a correct level autologous blood collection and intraoperative blood salvage is another option to cure such incidences.[10]

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