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Research Article

IMMUNOHEMATOLOGICAL EVALUATION OF HDFN CASES WITH DAT POSITIVITY IN A TERTIARY CARE CENTRE

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A R T I C L E I N F O

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A B S T R A C T

Hemolytic disease of the fetus and newborn (HDFN) is a condition in which fetal or newborn infants' RBCs have a shortened life span due to maternal antibodies. These antibodies cross the placenta and sensitize the fetal RBCs; they are usually IgG alloantibodies but on rare occasions can be IgG maternal autoantibodies..[1,2] Most common antibody anti-D followed by anti-c, -Jka, and -K, followed by anti-C, -s, -e, -cE, -Fya.[10] Blood grouping and Rh typing was done by tube technique. Polyspecific and Monospecific Direct Agglutination Test were performed by Column agglutination technique. Specificity of the antibody, elution studies were carried out using Glycine acid elution and Glycine acid /EDTA elution procedures. This present study has statistical significance between Severity of Direct Agglutination Test and the IgG1 subclass with hemolysis. Parity has a significant role in Rh HDFN. Acid Elution is highly effective in dissociating maternal antibodies by fetal RBCs which helps in identifying maternal alloantibodies. Glycine acid EDTA elution is useful for Phenotyping of the Fetal RBCs and transfusing Antigen Negative units to the affected neonates. Elution plays a major role in identifying Rh and other antibodies in neonates where maternal serum is unavailable, hence, institutional based algorithm for antenatal mothers.

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INTRODUCTION

Hemolytic disease of the fetus and newborn (HDFN) is a condition in which fetal or newborn infants' RBCs have a shortened life span due to maternal antibodies. These antibodies cross the placenta and sensitize the fetal RBCs; they are usually IgG alloantibodies but on rare occasions can be IgG maternal autoantibodies. This condition is more commonly called hemolytic disease of the newborn (HDN), but prefer the more accurate term, HDFN.[1,2] The hemolytic process varies from severe in utero hemolysis early in pregnancy, resulting in fetal death (often associated with hydrops fetalis), to a mild process that might not be noticeable until a day or more after the baby's birth. Sensitization of the baby's RBCs with maternal antibody does not necessarily lead to HDFN.[3]

The first antibody to be described as a cause of HDFN was anti-D. A positive DAT in a new-born result is due to transplacental transfer of IgG antibodies, which are present in maternal serum and directed against antigens on fetal and neonatal red blood cells (RBCs).

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Such antibodies may cause destruction of neonates' RBCs and shorten their life span, leading to clinical manifestations of HDN and various degrees of hyperbilirubinemia and anemia.[4] The factors that can lead to a positive DAT in neonates are mainly the maternal alloimmunization, ABO incompatibility between the new-born and the mother, and very seldom maternal autoimmune hemolytic anemia.[3] ABO incompatibility with a positive DAT is considered a major risk factor for the development of severe hyperbilirubinemia and neurotoxicity.[5,6] In contrast, some studies report that the positive DAT has only a poor predictive value for severe hyperbilirubinemia.[6-9]

Historically, the next most common antibody after anti-D was anti-E, followed by anti-c, -Jka, and -K, followed by anti-C, -s, -e, -cE, -Fya.[10] In 1964, Giblett reported that 93% of antibodies detected in sera of pregnant women were anti-D (or anti-C+D).[11] Kornstad *et al*., reported that in Norway, the occurrence of new cases of anti-D had fallen from 0.6% to 0.2%.[12]

Mollison *et al*., The commonest IgG red cell antibodies in human serum are anti-A and anti-B, although, relatively high concentrations are found only in group O subjects. Although ABO haemolytic disease is common, relatively few infants are severely affected; the proportion is higher in some populations

than in others. Haemolytic disease due to anti-D tends to be more severe than haemolytic disease due to anti-c.[13]

The direct antiglobulin test (DAT) should be performed in every neonates in whom the presence of hemolysis has been established. Although some exceptions to this rule might be considered, as when the diagnosis of a congenital hemolytic anemia is evident.

The DAT is a simple, quick, inexpensive test that yields useful information. A positive result on a DAT in a patient with hemolytic anemia does, of course, indicate that the most likely diagnosis is one of the immune hemolytic anemias. The predictive value of positive DAT is 83% in a patient with immune hemolysis, but only 1.4% in patients without immune hemolytic anemia.[14]

Severity of haemolysis was correlated with the number of antibodies bound to the RBC and the strength of DAT.[15,16] A positive DAT did not always mean decreased RBC survival. Many studies found out the relationship between the presence or absence of hemolysis and the DAT strength was highly statistically significant.[17,18] S S Das *et al*, from India also observed a significant correlation between strength of DAT and severity of hemolysis. The predictive value of a positive DAT was 83% in the patients with IHA, but only 1.4% in the patients without IHA.[19] Dorothy Dinesh in her study indicates that the sensitivity of a positive DAT for clinically significant HDN is 85%.[20] Huub H.vanRossum *et al* estimated the positive predictive value (PPV) calculated was 10% for DAT and eluate.[21] It should be emphasized that determination of the presence or absence of hemolysis, should logically precede the performance of the DAT. Therefore, interpretation of DAT must be done along with clinical history and other laboratory findings. Further evaluation of a positive DAT in a patient with clinical and laboratory evidence of haemolysis includes testing for clinically significant antibodies to RBC antigens and testing an eluate.[2]

Applications of Elution in HDFN

High sensitivity of both techniques (DAT and Elution) on detecting neonatal erythrocytes sensitized with anti-A and anti-B. Especially for A/O-incompatible pregnancies it appears that some degree of sensitization of neonatal erythrocytes with maternal IgG anti-A occurs regularly. "sub clinical" erythrocyte sensitization results in a low positive predictive value and specificity for both DAT as well as eluate screening. Screening for HDN by DAT results in many false positive results. In cases of ABO-incompatible pregnancies large percentages of positive neonatal DAT results are observed in the absence of clinical jaundice.[21] Micro positive DATs yielded a lower rate of new antibody detection (5.5%) than the combined groups of macroscopically positive DATs, 12.2% (p = 0.047). Eluate testing in the setting of micro positive DATs should not be a standard practice.[22] In Elution with Glycine acid/Elu-kit II in DAT positive cord samples, Antibodies were eluted from all DAT positive cord blood RBCs. Apart from ABO HDFN all other antibody mediated HDFN can be diagnosed antenatally. However, Elution can be of useful value in diagnosing clinically significant ABO HDFN and difficulty in obtaining maternal serum for other HDFN.[23]

MATERIALS AND METHODS

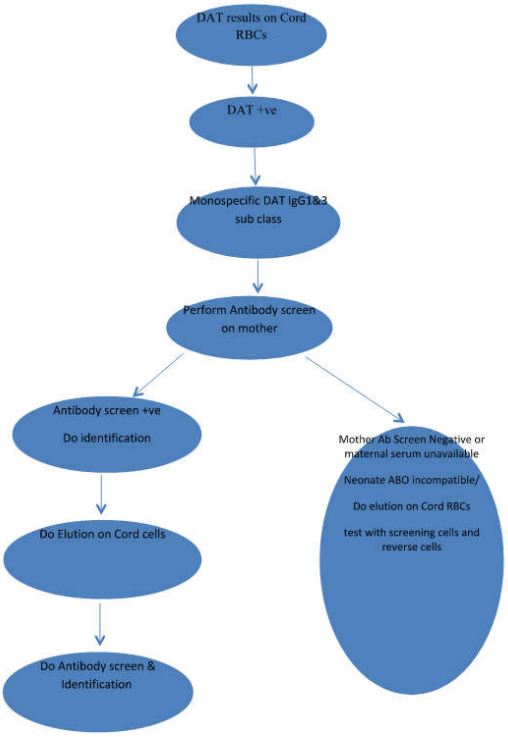
This study was carried out in the Department of Transfusion Medicine for the period of one year.

All samples were received for DAT in Ethylene Diamine Tetra Acetic acid (EDTA) tube/Vaccutainers. Blood grouping and Rh typing was done by tube technique. Polyspecific DAT was performed by CAT using LISS Coombs ID card "DAT IgG/C3d" which can detect IgG and C3d.

The blood samples of the patients', who could potentially be included in the study, were temporarily stored at room temperature 37^0C and samples were processed within 24 hours of collection.

Patients who had positive polyspecific DAT results were further evaluated by using monospecific Coombs card "IgG, IgM, IgA, anti C3d and anti C3c" which could detect the presence of anti-IgG, anti-IgM, anti-IgA, anti-C3d and anti-C3c. If monospecific cards are positive IgG, further IgG subtyping was performed using anti human globulin IgG1 and IgG3 by CAT from BIORAD and this was done in two dilutions of 1:1 and 1:100. Further, to find out the specificity of the antibody, elution studies were carried out using Glycine acid elution and Glycine acid /EDTA elution procedures.

Algorithm for Dat Positive Cord Red Blood Cells (HDFN)



RESULTS

In this study Total DAT positive HDFN cases were 21

Distribution of parity in HDFN

Out of 21 HDFN neonates, 17 Rh HDFN mothers, 14 mothers were Multi para and 3 were grand multi, in ABO HDFN mothers 2 were Primiparae and 1 was multipara and the 1 neonate with other HDFN mother was Grand multipara. The association between parity with Rh HDFN is statistically significant with a p value of < 0.0001. (p value < 0.05)

Correlation of Hyperbilirubinemia (TSB >15mg/dl) with HDFN

From a total of 21neonates, 16 (76%) had hyperbilirubinemia TSB >15mg/dl, in which 5 infants needed Exchange Transfusion and 16 were treated with phototherapy. The

remaining 5 Infants who had mild hyperbilirubinemia TSB < 15mg/dl needed no intervention. There is statistically significant correlation between hyperbilirubinemia with the management of HDFN and has a p value of < 0.000.

Correlation of HGB with severity of HDFN

Hemoglobin was assessed in patients with moderate to severe HDFN and mild HDFN by unpaired T test. The mean hemoglobin of moderate to severe hemolysis is 13.46gm/dl and of mild HDFN is 16.04gm/dl. There is statistically significant Correlation in hemoglobin between patients with moderate & severe hemolysis and mild hemolysis with p value <0.0013. (p value < 0.05)

Specificity of Monospecific DAT in HDFN

Out of 21 Cases of HDFN (100%) all of them had anti-IgG only in Monospecific DAT.

Correlation of IgG subclass with severity

Among 21 neonates with HDFN, 12 neonates who had IgG1 (9 were having moderate & severe and 3 mild HDFN); 5 neonates had IgG1 & IgG3 subtypes all of them had severe hemolysis, 3 neonates were having IgG3 subtype in which 2 had severe HDFN and 1 had mild HDFN; finally in the group of neither IgG1 nor IgG3 1 neonate had mild HDFN. There is a statistically significant correlation between the presence of IgG1, IgG1 and IgG3 antibodies and moderate & severe HDFN with a p value of < 0.012. (p value < 0.05)

Correlation of hemolysis with Rh HDFN and ABO HDFN:

Out of 17 Rh HDFN neonates, 15 had moderate to severe hemolysis and 2 had mild hemolysis. Out 3 ABO HDFN neonates in this present study 1(33%) neonate had severe HDFN and 2 (67%) neonates had mild HDFN. However, there is significant correlation between Rh HDFN and severity of hemolysis with a p value of 0.012. (p value < 0.05)

Acid Elution in HDFN

Among the 21 neonates acid elution was done in all the infants. 20 eluates were reactive and 1 eluate of ABO HDFN was nonreactive. In this study there is a statistically significant correlation between the DAT positive neonates and antibody identification in the eluate with a p value of <0.0001. (p < 0.05)

Glycine Acid EDTA Elution in HDFN

Out of 21 HDFN neonates all under gone Glycine Acid EDTA DAT was negative in all neonates and Rh phenotyping along with other specific phenotypes were identified. However, Kell antigens (K,k) were destroyed by this elution method.

DISCUSSION

DAT positivity in new-born is only due to HDFN either due to Rh incompatibility or due to ABO incompatibility between mother and fetus. The magnitude of the hemolytic disease of fetus and neonate (HDFN) is influenced by the immunoglobulin subclass, antibody levels and number of antigenic sites on the red cell. For diagnosing HDFN in ABO incompatible pregnancies, additional information such as neonatal haemolysis and high titres of antibody present in the mother could be helpful to improve the diagnostic accuracy.

Etiology

In our present study, Out of 21 cases of DAT positive HDFN cases received 17 (81%) were due to Rh, 3 (14%) were due to ABO HDFN and 1(5%) is due to others. Similarly in the study conducted by Dharmesh Chandra Sharma *et al* Rh incompatibility was the commonest cause of HDFN with 61 (55.5%) cases of RhD HDFN whereas ABO and other group HDFN cases were 30 (27.3%) and 19 (17.3%) respectively.[7]

Parity and HDFN

In our study among mothers of ABO HDFN 67% were Primiparae and 33% was multipara, while in 17 Rh HDFN 82%of those of Rh HDFN belonged to multipara and 18% were belonged to grand multi. Similarly in the study conducted by Ashutosh kumar *et al*., In neonates with ABO incompatibility, 37.5% were primi, and 42% were Multi para and the remaining were grand multi para, However in neonates with Rh incompatibility, 54% to grand multi and 36% belonged to multipara and only 9% were primi para.[24]

Correlation of Hyperbilirubinemia (TSB >15mg/dl) with HDFN

From a total of 21neonates, 16 (76%) with hyperbilirubinemia had TSB >15mg/dl, in which 5 infants needed ET and 16 were treated with phototherapy. The remaining 5 Infants who had mild hyperbilirubinemia TSB < 15mg/dl needed no intervention. Similarly in the study done by Swathi Chacham *et al* 41/48 (85%) had hyperbilirubinemia with TSB >15mg/dl all of them needed Phototherapy and/or ET.[25]

Mean HGB value in Moderate to Severe HDFN and mild HDFN patients

Mean hemoglobin was assessed in patients with moderate to severe HDFN and mild HDFN. The mean hemoglobin of moderate to severe hemolysis is 13.46gm/dl and of mild HDFN is 16.04gm/dl. Similarly Mollison *et al*,. stats that Moderately severe HDFN had a cord Hb concentration of 12.8 g/dl, and approximately 50% of the HDFN neonates had cord Hb concentrations of 14.5 g/dl or more, 30% had cord Hb values between 10.5 and 14.4 g/dl, and about 20% had Hb values of between 3.4 and 10.4 g/dl. The probability of survival diminishes as the cord Hb concentration falls. A level of 4 mg/dL or more of cord blood bilirubin (normal range, 0.7–3 mg/dL) or a rapidly rising bilirubin is suggested as an indication for exchange transfusion.[1] If the cord total bilirubin is less than 4–5 mg/dL and rising only slowly, phototherapy might suffice to correct the problem.[13]

Antibodies found in neonates with HDFN

Out of 17 Rh HDFN neonates Anti-D was found in 13 (76%) neonates, anti-D & anti-C was found in 2 (12%) neonates, anti-c was found in 2 (12%) neonates only. Among 3 ABO HDFN Anti-A was found in 1 Neonate and Anti-B was found in 2 neonates and among other blood group antigens anti-M was found in 1 neonate.

Transplacental passage of maternal IgG results in antibody coating of the fetal red cells. High alloantibody concentrations lead to a strong DAT, whereas low concentrations or binding affinity result in a weak or, by traditional methods, often negative DAT. A strong positive DAT is observed particularly with anti-D; however, the reaction is more variable with alloantibodies against other Rh antigens and antigens such as Kell, Duffy, Kidd, and MNSs, which are well expressed on fetal red cells.[31]

Correlation between Strength of DAT with severity of Rh HDFN

Out of 17 neonates with Rh HDFN 15 neonates with moderate to severe hemolysis had >2+ DAT strength and 2 neonates had <2+ as strength of DAT and presented with mild HDFN. There is significant correlation between Rh HDFN and severity of HDFN. Similarly in a letter to editor written by Mustafa Aydin *et al*, they stated that severity of Rh HDFN is well correlated with strength of DAT.[26]

Specificity of DAT in HDFN

Out of 21 cases of HDFN (100%) all of them had anti-IgG only in Monospecific DAT. Similarly in the study conducted by Pollock & Bowman IgG was the only antibody identified in cases of HDFN.[28]

IgG subtype of DAT in HDFN

Out of 21 HDFN cases, IgG1 was identified in (12) 52% of the neonates; IgG1 & IgG3 (5) were found in 29% of the neonates, IgG3 was found in (3)14% of the neonates and neither IgG1 nor IgG3 were seen in 5% (1) neonate. Similarly in a study done by Frankowska and Gorska, they found that 87.6% had IgG1 Rh antibodies, 23% contained IgG2 antibodies, 56.9% contained IgG3 antibodies, and 7.7% contained IgG4 antibodies.[28]

Correlation between IgG subclass and severity of HDFN

Out of 17 Rh HDFN 2 neonates had mild HDFN and 15 neonates had Moderate to severe HDFN. The distribution of IgG1, IgG1& IgG3 and IgG3 was 11 (65%), 3 (14%) and 3 (14%) respectively among the neonates with Rh HDFN. This present study has statistical significance between the subclass and severity of Rh HDFN. Similarly, Nance *et al*., in their study found that severe HDFN was associated with IgGl antibodies more frequently than with IgG3 antibodies.[29]

Glycine Acid Elution and Glycine Acid EDTA Elution in HDFN

Among the 21 neonates acid elution was done in all the infants. 20 eluates were reactive and 1 eluate of ABO HDFN was nonreactive. In this study there is a statistically significant correlation between the DAT positive neonates and antibody identification in the eluate. R.H. Finck *et al*., in their study found that antibodies eluted from cord blood RBCs was 100 percent (7 of 7). He also mentioned that apart from ABO HDFN all other antibody mediated HDFN can be diagnosed antenatally.[23] Out of 21 HDFN neonates all under gone Glycine Acid EDTA DAT was negative in and Rh phenotyping along with other specific phenotypes were identified. However kell antigens (K,k) were destroyed by this elution method,.

Antibodies found in neonates with HDFN by elution

Out of 17 Rh HDFN neonates Anti-D was found in 13 (76%) neonates, anti-D & anti-C was found in 2 (12%) neonates, anti-c was found in 2 (12%) neonates only. Among 3 ABO HDFN Anti-A was found in 1 Neonate and Anti-B was found in 2 neonates and among other blood group antigens anti-M was found in 1 neonate. Huub H.vanRossum *et al*., study explains the high sensitivity of both techniques (DAT and Elution) on detecting neonatal erythrocytes sensitized with anti-A and anti-B as the maternal serum can be used to detect the HDFN due to other blood group antigens.[21]

CONCLUSION

The most common cause of DAT positivity among neonates is Rh HDFN and ABO HDFN is second common one. This present study has statistical significance between Severity of DAT and the IgG1 subclass with hemolysis. Parity has a significant role in Rh HDFN. Acid Elution is highly effective in dissociating maternal antibodies from fetal RBCs, which helps in identifying maternal alloantibodies. Glycine acid EDTA elution is useful for Phenotyping of the Fetal RBCs and transfusing Antigen Negative units to the affected neonates. Elution is useful in diagnosing clinically significant ABO HDFN. Elution plays a major role in identifying antibodies in Rh and other HDFN neonates where maternal serum is unavailable. However apart from ABO HDFN all other antibodies are detected in maternal serum during the antenatal period, Hence an institution based algorithm for antenatal mother antibody screening and also for HDFN investigation will improve the early diagnosis of HDFN and planning management of the same.

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Conflict of interest

There is no Conflict of interest

Ethical approval

Ethical approval was obtained from concerned institutions.

Informed consent

Informed consent was obtained from all individual participants included in the study.

Table 1 Distribution of IgG subtypes

IgG Subtypes	Primi		Multi		Grand Multi		Total
	Mild	Severe	Mild	Severe	mild	Severe	
IgG1	0	0	2	7	1	2	12
IgG1 & IgG3	0	1	0	3	0	1	5
IgG3	0	1	1	1	0	0	3
Neither IgG1 nor IgG3	0	0	0	0	1	0	1

Table 2 Phenotype of the Neonates

Probable Phenotype	No. of neonates
D,C,c,e	12
D,C,c,E,e	01
D,c,e	05
D,c,E,e	02
D,C,c,e,M, N, s	01

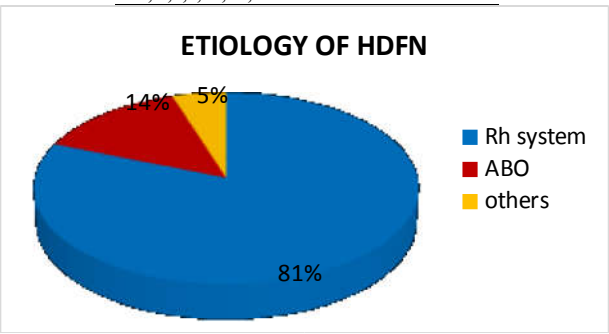

Fig 1

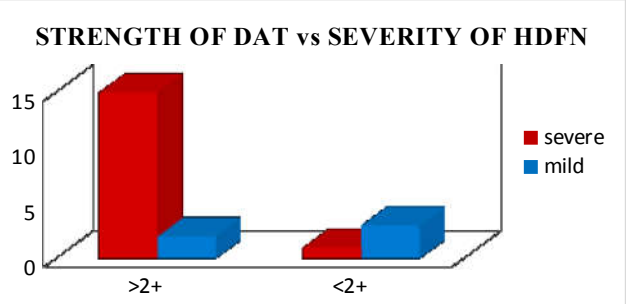

Fig 2

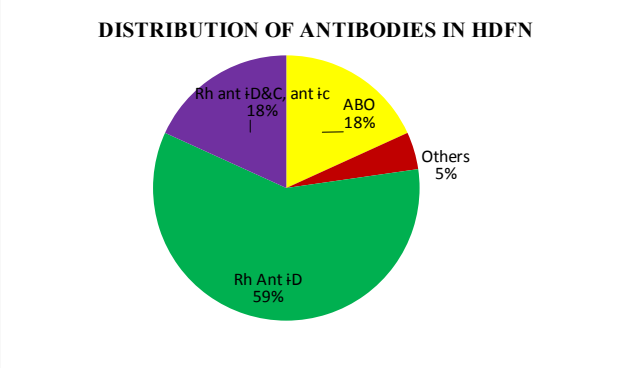

Fig 3

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