

Early Prediction of ABO HDN Using Immuno-Hematological and Biochemical Tests

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ABSTRACT

Objective: ABO hemolytic disease of the newborn (ABO HDN) is the most common cause of neonatal jaundice due to feto-maternal incompatibility. The objective of this study is to evaluate early predictors of ABO HDN using immuno-hematological and biochemical tests.

Materials and method: Newborns with blood group A/B born to group O mothers were prospectively enrolled in the present study. Direct antiglobulin test (DAT) and cord blood bilirubin (CBB) were performed. Total serum bilirubin (TSB) was measured at 72 hours of life. Serum anti-A/B IgG titers of maternal samples were measured. For the statistical analysis, Microsoft Excel 2013 (Microsoft Corporation, NY, USA) and SPSS statistical computer software (version 22) were used. To find the efficacy and cut-off values for various predictors of neonatal jaundice, receiver operating characteristic (ROC) curve analysis was performed. To evaluate the relative importance of predictors for phototherapy (PT), logistic regression was used.

Results: A total of 200 ABO incompatible mother-neonate pairs were included in the present study. The incidence of ABO HDN was 10% (n=20). The number of DAT positive cases was 38 (19%). Regression analysis showed a significant influence of antibody titre, CBB and DAT on probability of phototherapy. Antibody titers ≥ 512 predicted severe hyperbilirubinemia with 80% sensitivity and 83% specificity; CBB ≥ 2.75 mg/dL had 85% sensitivity and 83% specificity.

Conclusion: High risk ABO-incompatible neonates could be identified at birth using maternal antibody titers, DAT and CBB. Early diagnosis with enhanced surveillance will help in optimum utilization of healthcare.

Keywords: ABO-antibody, ABO hemolytic disease of newborn, hemolytic disease of fetus & newborn (hdfn), DAT, cord blood bilirubin, jaundice neonatal, Coombs test, blood group incompatibility, ABO antibody titer.

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INTRODUCTION

Hyperbilirubinemia occurs in more than 60% of healthy infants during the first week of life, with peaks usually being noted on days 3-5, and resolves within 7-10 days of age (1-3). Early discharge of newborns on days 1-2 after birth makes it more difficult to identify those who are at risk of severe hyperbilirubinemia and also leads to a higher readmission rate. The American Academy of Pediatrics (AAP) enlists blood group incompatibility and positive direct antiglobulin test (DAT) as a major risk factor for severe hyperbilirubinemia (4).

ABO hemolytic disease of the newborn (ABO HDN) is currently the most common cause of neonatal jaundice due to feto-maternal blood group incompatibility (5). Approximately 50% of babies with ABO HDN do not require any treatment. Of the remaining 50% newborns, half of them become severely jaundiced, and 90% of them will die without treatment, while 10% will develop kernicterus (6). It was in every 10th newborn that ABO incompatibility manifested as a hemolytic disease requiring treatment. The AAP recommends that follow up should be provided in two to three days of discharge if the neonate is discharged within 48 hours after birth (4). Compliance with this advice may not be easy, particularly in rural or lower socioeconomic areas. Neonatal hyperbilirubinemia owing to ABO incompatibility is also considered to be the most common cause of readmission within initial days of discharge (5). The prediction of significant hyperbilirubinemia at birth offers an attractive option to identify neonates at risk and reduces hospital stay for healthy neonates.

Screening practices for ABO HDN vary globally and are influenced by the healthcare infrastructure, regional healthcare priorities and resource availability. Advanced immunohematological tests, including maternal antibody titration, direct antiglobulin test (DAT) and elution testing, are essential for accurate diagnosis. However, these tests are often inaccessible in lower socioeconomic regions due to limited resources and inadequate laboratory facilities. Consequently, ABO-HDN is frequently underreported and misdiagnosed as physiological jaundice during its early stages, which delays appropriate treatment and management.

This study aimed to determine the incidence of ABO HDN and to provide early diagnostic evidence for predicting the occurrence of neonatal ABO hemolytic disease using biochemical and immuno-hematological tests. □

MATERIALS AND METHODS

This prospective observational study was conducted in the Department of Transfusion Medicine of a tertiary care hospital and teaching institute in Mumbai, India, over a period of one year. The study was approved by the Institutional Ethical Committee. Two hundred healthy full-term (38 weeks of pregnancy) A or B blood group newborns born to blood group O mothers were prospectively enrolled in our study after taking their mothers' informed consent. Confidentiality of participant data was maintained at all levels. Demographic details were collected from the mother. Neonates with a hemolytic condition other than ABO incompatibility, positive maternal history of autoimmune diseases, low birth weight neonates (<2.5 kg), significant illness requiring neonatal intensive care unit (NICU) admission, major congenital malformations and unavailability of the sample were excluded from the study.

Pregnant women admitted for labour were routinely tested for ABO and Rh blood grouping (tube technique) and antibody screening [indirect antiglobulin test (IAT)] by column agglutination technique using the commercially available polyspecific AHG cassette from Bio-Rad Laboratories, CA, USA) in our center. O Rh 'D' positive and IAT negative maternal blood samples were preserved in view of further testing. As a routine protocol, cord blood samples were tested for ABO and Rh blood groups. For babies born to O positive mothers whose blood group was found to be A/B Rh 'D' positive, the direct antiglobulin test (DAT) by column agglutination technique and cord blood bilirubin (CBB) estimation were done. Total serum bilirubin (TSB) was measured at 72 hours of life. For bilirubin estimation, Diazo method was performed in the Biochemistry department. Maternal serum anti-A and anti-B IgG titers were measured by the tube technique using dithiothreitol (DTT) (7). Significant hyperbilirubinemia was defined as total serum bilirubin (TSB) was > 95th percentile on a TSB nomogram (4). For this study purpose, TSB $\geq$ 15 mg/dL

at 72 hours of life was considered significant hyperbilirubinemia and ABO incompatible newborns born to blood group O mothers having significant hyperbilirubinemia were considered to have ABO HDN.

For the statistical analysis, Microsoft Excel 2013 (Microsoft Corporation, NY, USA) and SPSS statistical computer software (version 22) were used. Comparison of group differences for quantitative variables was calculated using independent t-test and categorical variable by Chi-square test. Correlations between quantitative variables were computed by Pearson's correlation coefficient. To find the efficacy and cut-off values for various predictors of neonatal jaundice, receiver operating characteristic (ROC) curve analysis was done. To evaluate the relative importance of predictors for phototherapy (PT) logistic regression was used. A logarithmic scale was used for antibody titre in tests and regressions. All tests were two-sided. P-values ≤ 0.05 were considered significant. $\square$

RESULTS

A total of 2184 O Rh 'D' positive women were delivered during the study period. Among these, 394 (18%) had neonates with blood group A or B. Two hundred mother-neonate pairs were enrolled in the present study after fulfilling the inclusion and exclusion criteria. One hundred and ninety-four mother-neonate pairs were excluded for various reasons as follows: 82 due to lack of consent, 55 for low birth weight, three

for congenital malformations, 17 for NICU admission, 24 for non-availability of samples and 13 for other causes (Figure 1). Among study population, O-A group incompatibility accounted for 46% (n=92) and O-B group incompatibility for 54% (n=108). Among male subjects, 23% (n=46) had O-A and 34% (n=68) O-B incompatibility, while among female subjects both incompatibilities were found in 23% (n=46) and 20% (n=40) of subjects, respectively, with a P-value of 0.06. Primigravida had a significantly

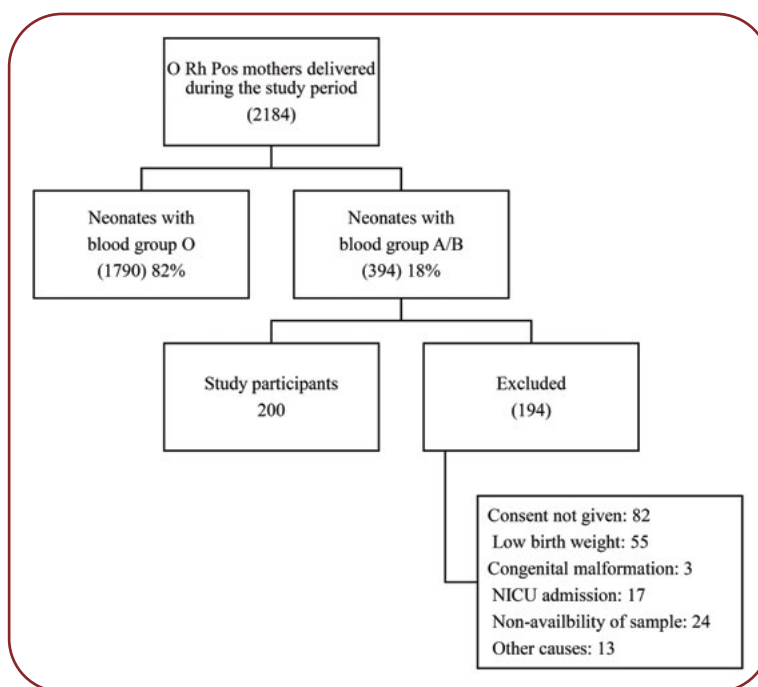


FIGURE 1. Inclusion and exclusion algorithm

Characteristics	Total (n = 200)	O-A incompatibility 92 (46%)	O-B incompatibility 108 (54%)	P-value
Male n (%)	114 (57%)	46 (23%)	68 (34%)	0.06
Female n (%)	86 (43%)	46 (23%)	40 (20%)	
Primigravida n (%)	120 (60%)	46 (23%)	74 (37%)	0.008*
Multigravida n (%)	80 (40%)	46 (23%)	34 (17%)	
Birth weight (kg) (mean $\pm$ SD)	2.85 $\pm$ 0.31	2.86 $\pm$ 0.31	2.84 $\pm$ 0.29	0.57
Cord blood bilirubin (mg/dL) (mean $\pm$ SD)	2.26 $\pm$ 0.74	2.27 $\pm$ 0.70	2.25 $\pm$ 0.76	0.83
Cord hemoglobin (gm %) (mean $\pm$ SD)	15.58 $\pm$ 1.14	15.58 $\pm$ 1.13	15.58 $\pm$ 1.15	0.90
Antibody titer geometric mean (min-max)	247 (16-2048)	286 (16-1024)	213 (16-2048)	0.11
Bilirubin at 72 hours (mg/dL) (mean $\pm$ SD)	11.33 $\pm$ 2.90	10.82 $\pm$ 2.77	11.76 $\pm$ 2.95	0.02*

* P-value ≤ 0.05 is considered statistically significant.

TABLE 1. Baseline characteristics of study population

Characteristics	DAT positive 38 (19%)	DAT negative 162 (81%)	P-value	Significant hyperbilirubinemia 20 (10%)	Non-significant hyperbilirubinemia 180 (90%)	P-value
Blood group A n (%)	18 (19.56)	74 (80.43)	0.85	08 (8.69)	84 (91.3)	0.64
Blood group B n (%)	20 (18.51)	88(81.48)		12(11.11)	96(88.89)	
Birth weight in kg (mean $\pm$ SD)	2.86 $\pm$ 0.29	2.85 $\pm$ 0.3	0.88	2.84 $\pm$ 0.28	2.85 $\pm$ 0.3	0.86
Cord blood bilirubin (mg/dL) (mean $\pm$ SD)	2.9 $\pm$ 0.84	2.11 $\pm$ 0.62	0.000*	3.48 $\pm$ 0.51	2.12 $\pm$ 0.62	0.000*
Bilirubin at 72 hours (mg/dL) (mean $\pm$ SD)	13.54 $\pm$ 4.07	10.8 $\pm$ 2.28	0.000*	16.85 $\pm$ 2.25	10.71 $\pm$ 2.25	0.000*
Antibody titer geometric mean	506 $\pm$ 491	186 $\pm$ 221	0.000*	755 $\pm$ 539	190 $\pm$ 221	0.000*
Phototherapy n (%)	17(85)	03(15)	0.000*			

*P-value $\leq$ 0.05 is considered statistically significant. DAT=direct antiglobulin test.

TABLE 2. Clinical characteristics of neonate in relation to direct antiglobulin test and phototherapy

higher rate of O-B incompatibility compared to multigravida ($P = 0.008$). Birth weight, cord blood bilirubin, cord hemoglobin and antibody titer levels showed no significant differences between the study groups. However, bilirubin levels at 72 hours were significantly higher in the O-B incompatibility group (P -value 0.02) (Table 1).

The incidence of ABO HDN was 10% ($n = 20$). In the present study, all neonates with ABO HDN received PT. No baby underwent exchange transfusion or IVIG treatment. Male subjects (10.52%, $n = 12$) were slightly more affected than female ones (9.30%, $n = 8$) ($p = 0.336$). Of the 20 newborns with significant hyperbilirubinemia, eight subjects were A Rh 'D' positive and 12 subjects B Rh 'D' positive. Bilirubin at 72 hours was significantly higher in blood group B (11.76 mg/dL) neonates than blood group A ones (10.82 mg/dL) ($p = 0.02$). No significant difference was found in antibody titer and CBB between blood group A and B neonates.

The clinical characteristics of neonates in relation to significant hyperbilirubinemia requiring PT and DAT status are summarised in Table 2. Among the 200 participants, 19% ($n = 38$) were DAT positive and 81% ($n = 162$) DAT negative. Blood group A and B distributions were similar between DAT positive and negative groups, with no significant differences being found. Birth weight showed no significant differences across groups. However, cord blood bilirubin and bilirubin levels at 72 hours were significantly higher in DAT positive and PT groups, indicating a higher risk of jaundice. Antibody titers were also significantly higher in DAT positive and PT groups.

Titer	DAT positive n (%)	Phototherapy n (%)	Total n (%)
≤ 64	8 (9.3)	0 (0)	86 (43)
128	4 (11.11)	2 (5.55)	36 (18)
256	4 (12.5)	2 (6.25)	32 (16)
512	14 (43.75)	8 (25)	32 (16)
1024	6 (50)	6 (50)	12 (6)
2048	2 (100)	2 (100)	02 (01)
Total	38 (19)	20 (10)	200

DAT=direct antiglobulin test.

TABLE 3. Relationship between antibody titer, direct antiglobulin test and phototherapy

The majority of neonates requiring PT were DAT positive.

The relationship between maternal antibody titer with DAT and requirement of PT is shown in Table 3. Among the study population, 19% ($n = 38$) were DAT positive and 10% ($n = 20$) required PT. As the titer levels increased, the proportion of DAT positive and neonates requiring PT also increased, which indicated a strong correlation between high antibody titers and the need for clinical intervention. The number of neonates receiving PT increased abruptly from antibody titers ≥ 512 . We found a moderate correlation of PT with CBB ($r = .55$, $P < .00$) and DAT ($r = .56$, $p < .00$). We also found a moderate correlation between antibody titer and phototherapy ($r = .43$, $p < .00$).

Logistic regression analysis showed that there is a significant influence of CBB, antibody titre and DAT on the probability of neonate receiving phototherapy [$\chi^2(5) = 89.09$, $p < 0.001$]. The model explained 75.2% variance in phototherapy (Nagelkerke R^2) and was able to identify 98% of cases accurately. The sensitivity and specificity of the model was 80% and 100%, respectively. The results showed that for every unit increase in

Characteristics	B (95% CI)	SE (B)	Wald chi-square test	Odds ratio
Log titre	2.954 (1.76, 208.56)*	1.217	5.891**	19.192
Cord DAT	-2.229 (.02, .58)	.863	6.669†	.108
Cord blood bilirubin	2.578 (3.02, 57.37)	.751	11.799††	13.176
Cord hemoglobin	-.018 (.34, 2.85)	.543	.001	.982
Birth weight	-.487 (.02, 19.15)	1.755	.077	.615
Constant	-13.903	10.446	1.771	.000

CI=confidence interval; SE=standard error; DAT=direct antiglobulin test.

Omnibus test: $\chi^2(5) = 89.09$, $p < .001$; $R^2 = .359$ (Cox & Snell); .752 (Nagelkerke).

*95% CI for the exponentiation of the B coefficient [Exp(B)]; ** $p < .05$; † $p < .01$; †† $p < .001$.

TABLE 4. Results of logistic regression analysis

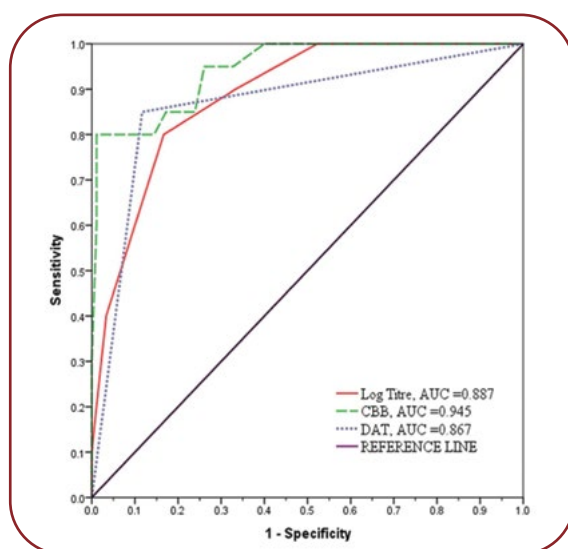


FIGURE 2. predicting the risk of ABO HDN requiring phototherapy (PT). Maternal antibody titer (log), CBB and DAT as test variables and PT group as state variable (Reference no PT group); ROC=receiver operating characteristic curve; AUC=area under curve.

log antibody titer and CBB, the odds for receiving PT were 19.19 and 13.17 times, respectively (Table 4). Sensitivity and specificity of maternal IgG anti-A/B titers for predicting significant hyperbilirubinemia are shown in the form of a ROC curve (Figure 2). Titers of ≥ 512 showed 80% sensitivity and 83% specificity to predict significant hyperbilirubinemia. Similarly, $CBB \geq 2.75$ mg/dL predicted significant hyperbilirubinemia with 85% sensitivity and 83% specificity (Figure 2). □

DISCUSSION

The diagnosis of ABO HDN depends on clinical, serological and biochemical findings in the newborn. Early diagnosis and intervention at the proper time is necessary to prevent its adverse outcome. We found that in ABO-incompatible neonates there was a strong association

between the level of maternal IgG antibody titre, CBB, DAT and significant hyperbilirubinemia.

In the present study, the incidence of ABO incompatibility and ABO HDN was 18% and 10%, respectively, which was in accordance to previous studies (8, 9). We did not find a significant difference between A and B group neonates with regard to antibody titer, CBB, DAT and PT. However, we found a significantly high 72-hour bilirubin level in blood group B neonates. The literature is inconsistent about the degree of hemolysis and incidence and severity of hyperbilirubinemia among O-A and O-B pairs (8, 10).

Maternal serum IgG anti-A (B) titer is one of the important early predictors of ABO HDN that is used as one of the prenatal diagnostic tests. Our study showed that the percentage of ABO HDN gradually escalated with increasing maternal IgG anti-A/B titer, which has been also proved by the correlation analysis. We found that antibody titer levels ≥ 512 markedly predicted an increased risk of ABO HDN, which was in accordance with the results of a meta-analysis done by Li P (11). Different authors have used different cut-off values of antibody titer for predicting significant hyperbilirubinemia. Mahfood *et al* identified anti-A/B antibody titers ≥ 64 as risk factors for HDN, while Bakkeheim *et al* demonstrated that titers ≥ 512 had 90% sensitivity and 72% specificity for predicting severe hyperbilirubinemia (12, 13). We also found that neonates whose mothers had antibody titers of ≥ 512 were 19 times more likely to receive PT compared to those with titers of < 512 . A similar study done by N Kadri revealed that when maternal antibody titers were > 256 , the risk of developing potential jaundice became 18 times higher (14). This variability in cut-off titers may be attributed to differences in study populations and timeline of sampling and methodology of antibody titer. Hence, the need for institutional

policy to define the cut-off value becomes more important.

CBB measures in utero hemolysis and can be used as biomarkers for significant hyperbilirubinemia secondary to HDN. Our study showed that CBB was a useful predictor of subsequent hyperbilirubinemia. We found that cut-offs of $\text{CBB} \geq 2.75 \text{ mg/dL}$ had 85% sensitivity and 83% specificity for significant hyperbilirubinemia. We also found a strong positive correlation between PT and CBB. A prospective study conducted by Satrya *et al* found that CBB cut-off values of $\geq 2.54 \text{ mg/dL}$ had 90% sensitivity and 85% specificity (15). Similarly, Calkins *et al* reported a cut-off value of 2.05 mg/dL with 80% sensitivity and 78% specificity for predicting phototherapy (16). Different authors have used different CBB cut-off values for prediction of significant hyperbilirubinemia. This variability can be due to variation in study populations, laboratory individuality, etc.

Direct antiglobulin test is one of the important diagnostic tests for immune HDN (17). It is generally weakly positive or negative, so the interpretation of DAT results in ABO HDN is usually difficult (18). A negative DAT does not rule out ABO HDN. Some institutions have implemented routine DAT as a cost-ineffective strategy, but clinical guidelines do not support it. The AAP recommends that if the maternal blood group is O Rh-positive, testing cord blood for the infant's blood group and DAT is optional (4). In the present study, the incidence of DAT positivity was 19%. Previous studies reported an incidence of DAT positivity of about 5-31% in ABO-incompatible neonates (18, 19). The risk of significant hyperbilirubinemia increases with positive DAT in ABO-incompatible neonates. In the present study, 85% of all neonates who received PT had a positive DAT. On logistic regression, we ob-

served that DAT positivity significantly was influencing the need for phototherapy. However, Dinesh *et al* found a positive predictive value of DAT as low as 23% (19). CBB, DAT and antibody titer can be readily available, low cost and non-invasive for the neonate. These screening test setups will be an ideal option for early prediction of ABO HDN.

The limitations of our study included the small sample size and the facts that preterm neonates were excluded from the study and patients were not followed-up on the long-term, so that other complications might have been missed. Therefore, larger studies are required to confirm the current findings and applicability of these screening tests for clinical use.

CONCLUSIONS

In a nutshell, management of ABO HDN entails coordination between clinicians (obstetricians and pediatricians) and transfusion medicine specialists to devise a standard protocol for long term benefits of neonates. If the maternal antibody titers were ≥ 512 and $\text{CBB} \geq 2.75 \text{ mg/dL}$, the risk for having ABO HDN in the neonate rose 19 and 13 times, respectively. Active antenatal screening with close follow-up of high titre group O mothers may be recommended. The neonates at risk of jaundice should be identified at birth and kept under observation for the occurrence of jaundice and prompt intervention. Early disease prediction and timely intervention will lead to an optimum utilization of healthcare resources and to reduced healthcare costs. This will also help the administrators to plan the infrastructure required for advanced immunohematology testing. $\square$

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