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Original Article

The clinical value of serum bilirubin, hemoglobin, and reticulocyte combined with immunohematological tests in the diagnosis of ABO neonatal hemolytic disease

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ABSTRACT

This study aims to investigate the clinical application value of serum total bilirubin, hemoglobin (Hb) and reticulocyte percentage (Ret%) combined with immunohematological tests in the diagnosis of ABO hemolytic disease of the newborn (ABO-HDN). A retrospective analysis of 503 neonatal blood samples of mothers with RhD(+) blood type O and neonates with RhD(+) blood type A/B, admitted to the Third Xiangya Hospital of Central South University from March 2020 to September 2021, was conducted. HDN was investigated with the elution test, indirect antiglubin test (IAT), and direct antiglubin test (DAT). The positive rate of the immunohematological tests for neonatal hemolysis, combined with the detection of serum total bilirubin, Hb and Ret%, was significantly higher than that of immunohematological tests for neonatal hemolysis alone (P<0.05). The elution test can be used as a gold standard to diagnose HDN. Neonates with blood type A had a higher probability of ABO hemolysis than those with blood type B. This study suggests that serum bilirubin, Hb, and Ret% detection combined with immunohematological tests can improve the detection rate of ABO-HDN.

Keywords: ABO hemolytic disease of the newborn, serum total bilirubin, direct antiglubin test, indirect antiglubin test, elution test

INTRODUCTION

Hemolytic disease of the newborn (HDN), also known as fetal erythroblastic disease (erythroblastosis fetalis)[1], is mainly due to maternal and fetal blood group incompatibility. The mother produces natural immunoglobulin G (IgG) subtype antibodies against fetal red blood cell (RBC) antigens inherited from the father, leading to alloimmunization and neonatal hemolysis[2–3]. HDN caused by ABO blood group antibodies is most common in Asian populations, followed by the Rh blood group system. Erythrocytes bound with IgG antibodies are destroyed by macrophages in the spleen and other reticuloendothelial tissues[4], resulting in extravascular hemolysis, with clinical manifestations in newborns including moderate jaundice, anemia, edema, and life-threatening bilirubin encephalopathy of the nervous system. Therefore, early diagnosis and timely treatment of HDN are crucial. If timely and effective

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treatments are not implemented, HDN can easily lead to the destruction of liver function and other complications, posing a serious threat to the health and life safety of newborns[5]. In clinical practice, immunohematological tests including the direct antiglobulin test (DAT), indirect antiglubin test (IAT), and antibody elution test, combined with serum total bilirubin and routine blood tests, are primarily used for the early diagnosis of HDN. This study retrospectively analyzed and reported a total of 503 cases of maternal type O RhD(+) blood and neonatal type A/B RhD(+) blood collected at our hospital.

MATERIALS AND METHODS

General information

A total of 503 newborns with suspected ABO-HDN admitted to the Third Xiangya Hospital of Central South University from March 2020 to September 2021 were included. Among them, there were 301 males and 202 females with ages ranging from 1 hour to 10 days and an average age of (5.02±2.25) days. This study was approved by the Ethics Committee of the Third Xiangya Hospital of Central South University (2021-5307). The total bilirubin level of the newborns ranged from 128 to 336 μmol/L. The maternal blood group was determined to be type O RhD(+). There were 286 cases of type A RhD(+) and 217 cases of type B RhD(+) in ABO-HDN newborns.

Inclusion and exclusion criteria

Inclusion criteria: (1) All cases were in accordance with the Expert Consensus on Hemolysis Detection and Test Results Reporting of Clinical Specimens[6]; (2) The maternal blood type was O RhD(+) and incompatible with the neonatal blood type[6]; (3) All newborns had clinical manifestations of HDN, such as yellowish discoloration of the complexion, skin, and sclera; (4) All were accompanied by jaundice, anemia, hepatosplenomegaly, and other clinical symptoms; (5) The neonates' medical records were complete and detailed.

Exclusion criteria: (1) Premature infants with liver and biliary system disease; (2) Newborns with other immune system diseases or other blood related diseases; (3) Neonates with incomplete clinical data.

Maternal and fetal blood group detection

The blood groups of forward and reverse typing were detected with microcolumn agglutination (BioRad, USA) according to the recommended protocols. Briefly, the whole blood specimens were centrifuged at 900–1000 g for 5 minutes, and the RBCs were collected and used to prepare a 0.8% RBC suspension for forward typing. The serum (50 μL) was used for reverse typing.

Immunohematological detection

In the reference[7], a specific detection method for blood samples in newborns with HDN was provided in the recommended scheme of postpartum immune hematology tests of HDN. Neonatal ABO incompatibility hemolytic disease needs to be determined by combining the three results of neonatal hemolysis (***Table 1***)[7].

Table 1 **Diagnosis of hemolytic disease of the newborn by three hemolysis tests[7]**

DAT	IAT	Elution test	Result
Negative	Negative	Negative	No HDN
Positive	Negative	Negative	Suspected HDN
Negative	Positive	Negative	Suspected HDN
Negative	Negative	Positive	ABO-HDN
Positive	Negative	Positive	ABO-HDN
Positive	Positive	Negative	ABO-HDN
Negative	Positive	Positive	ABO-HDN
Positive	Positive	Positive	ABO-HDN

DAT: direct antiglubin test; IAT: indirect antiglubin test; HDN: hemolytic disease of the newborn.

Serum total bilirubin, hemoglobin (Hb), and reticulocyte detection

Serum total bilirubin was measured by a Biochemical Analyzer (model ADVIA2400, Siemens, Germany) with a normal reference range of 3.42–20.5 μmol/L. Hb (normal reference range: 150–210 g/L) and reticulocyte percentage (Ret%) (normal reference range: 2.0%–6.0%)[8] were detected by Automatic Hematology Analyzer (BC6800Plus, Mindray, Shenzhen, China).

Statistical analysis

SPSS 23.0 statistical software was used for relevant data processing. Measurement data were expressed as

mean ± standard deviation (SD), and the *t*-test was used. Enumeration data were expressed as frequency and percentage (%) using the Chi-square (x^2) test. $P<0.05$ indicated statistically significant differences.

RESULTS

Detailed results and clinical situation of immunohematological tests for hemolysis of newborn

Among the 503 newborns with suspected ABO-HDN who underwent immunohematological tests for hemolysis of the newborn, 18 (3.58%) were tested positive in all three tests: IAT, elution test, and DAT; 81 (16.10%) were tested positive in two of the tests; and 57 (11.33%) were tested positive in one test. Combined with the results of immunohematological tests for neonatal hemolysis, a total of 156 ABO-HDN positive cases were finally confirmed, with a detection rate of 31.01%. Comparing the positive results of two tests and one test, the cases with positive immunohematological tests had significantly increased total bilirubin content in the serum, while the total bilirubin content in serum in cases with positive hemolysis tests was significantly increased compared with cases with negative hemolysis tests ($P<0.001$). Compared with hemolysis test negative newborns, the serum total bilirubin content of newborns with positive hemolysis test was significantly increased, and the serum total bilirubin content of newborns with positive hemolysis test was the highest (***Table 2***). The mean hemoglobin contents in the cases with positive results from two tests and one test were lower than those in cases with negative hemolysis tests (131.11±15.32 *vs*. 165.23±11.26), as well as the neonatal anemia standard of 145 g/L. However, the Ret% was significantly higher in cases with one or two positive tests than in cases with negative hemolysis tests (5.24±1.63 *vs*. 3.62±1.21) (***Table 3***).

Table 2 **Results of immunohematological tests, serum total bilirubin, and Hb in 503 newborns**

Group	DAT	IAT	Elution test	Judgment result	Cases (%)	Total bilirubin ($\bar{x}$±s, μmol/L)	Hb (g/L)	Ret%
1	+	+	+	Positive	18 (3.58)	291.94±38.24	105	6.33
2	+	+	−	Positive	2 (0.40)	253.14±20.86	124	5.24
3	+	−	+	Positive	9 (1.79)	252.94±21.46	128	5.46
4	+	−	−	Doubtful	0	0	0	0
5	−	+	+	Positive	70 (13.92)	251.00±22.12	132	5.18
6	−	+	−	Doubtful	0	0	0	0
7	−	−	+	Positive	57 (11.33)	242.58±20.56	139	4.27
8	−	−	−	Negative	347 (68.99)	130.43±27.92	165	3.62

Hb: hemoglobin; DAT: direct antiglubin test; IAT: indirect antiglubin test; Ret%: reticulocyte percentage.

Table 3 **Comparison of serum total bilirubin, Hb, and Ret% in newborns with HDN ($\bar{x}$±s)**

Group	*n*	Total bilirubin (μmol/L)	Hb (g/L)	Ret%
HDN positive	156	252.79±30.43	131.11±15.32	5.24±1.63
HDN negative	347	130.43±27.92	165.23±11.26	3.62±1.21
t value		44	27.97	12.41
P value		<0.001	<0.001	<0.001

Hb: hemoglobin; Ret%: reticulocyte percentage; HDN: hemolytic disease of the newborn.

Comparison of detection rates of immunohematological tests for hemolysis of newborn

Among the 156 newborn cases with positive hemolysis of newborn, 143 (91.67%) were tested positive in the RBC antibody elution test, 90 (57.69%) were tested positive in the serum free antibody test, and 29 (18.59%) were tested positive in the DAT. The detection rate of antibody elution test was significantly higher than that of free antibody test and erythrocyte DAT, and the differences were statistically significant ($P<0.05$). Details are shown in ***Table 4***.

ABO-HDN positive rate detected by serum total bilirubin, Hb, and Ret% combined with immunohematological tests in hemolysis of newborn

156 newborn cases were tested positive for hemolysis of the newborn by immunohematological tests alone, while 198 cases were tested positive when combining immunohematological tests with

measurements of serum total bilirubin, Hb, and Ret%. The positive detection rate of ABO-HDN detected by immunohematological tests combined with serum total bilirubin, Hb, and Ret% was significantly higher than that detected by immunohematological tests alone (P=0.006) (***Table 5***).

Table 4 **Comparison of detection rates of immunohematological tests in newborn**

Test method	Number of cases	Positive cases	Negative cases	Detection rate (%)
DAT	156	29	127	18.59
IAT	156	90	66	57.69
Elution test	156	145	11	92.95

DAT *vs*. IAT (X^2=50.549, P<0.001); DAT *vs*. elution test (X^2=206.507, P<0.001); IAT *vs*. antibody elution test (X^2=77.022, P<0.001); DAT: direct antiglubin test; IAT: indirect antiglubin test.

Table 5 **ABO-HDN positive rate detected by three hemolysis tests combined with serum total bilirubin, Hb, and Ret% in newborns**

Test method	Number of cases	Positive cases	Positive detection rate (%)	P value (X^2 value)
Immunohematological tests	503	156	31.01	
Immunohematological tests combined with serum total bilirubin, Hb, and Ret%	503	198	39.36	0.006 (7.689)

HDN: hemolytic disease of the newborn; Hb: hemoglobin; Ret%: reticulocyte percentage.

DISCUSSION

At present, immunohematological tests are clinically important indicators for the early diagnosis of ABO-HDN, including the DAT, IAT, and RBC antibody elution test. The results showed that the positive rate of antibody elution test, IAT and DAT was 98.72%, 57.69%, and 18.59%, respectively. The detection rate of antibody elution test was significantly higher than that of IAT and DAT.

The DAT is used to detect whether there is sensitization of IgG antibodies from the mother in the RBCs of newborns, but due to the small number of antigens carried by the RBCs of newborns (only 1/4–1/2 of the number of RBCs of adults), the sites at which they can bind to IgG antibodies are limited, and IgG antibodies may also be partially neutralized after entering the newborn[9]. In this study, 29 patients who had been confirmed as hemolysis-positive had positive direct antibody test with a detection rate of only 18.59%. IAT is applied to detect free IgG antibodies in neonatal plasma. These antibodies may bind to neonatal RBCs and lead to immune destruction of neonatal RBCs[10]. Therefore, an IAT can be effectively used for the dynamic observation of therapeutic effects of ABO-HDN. Xu *et al*.[9] and Cai *et al*.[11] both detected the positive rate of IAT to be around 70%, but in this study, the detection rate of IAT was 57.69%, slightly lower than the average level. Both the DAT and IAT are easily affected by the number and structure of antigens on RBCs and the level of RBCs in children, resulting in an instable detection rate[12]. The elution test is the most important basis for the diagnosis of ABO-HDN, and a single positive can be taken as a diagnosis for HDN. In this study, the detection rate of elution test was as high as 98.72%, and the detection rate of elution test even reached 100% in the studies by Huang *et al*.[3], and Xu *et al*[9]. Among the immunohematological tests for hemolysis of the newborn, the elution test has the highest detection rate, sensitivity, and specificity, and can be used as a confirmation test for ABO-HDN.

Serum total bilirubin level is also an important indicator for the diagnosis of ABO-HDN and the development of hyperbilirubinemia. Studies have shown that HDN is the leading cause of severe neonatal hyperbilirubinemia, despite differences in the frequency of ABO-HDN and Rh-HDN[13]. ABO-HDN can lead to the destruction of a large number of RBCs when the level of bilirubin exceeds what the liver can process, so that the total bilirubin level in the serum continues to increase, which in turn causes neonatal hyperbilirubinemia (NHB)[14]. Severe NHB can cause hyperbilirubinemia encephalopathy, with a high mortality rate of 21.4%, while survivors may be left with varying degrees of neurological sequelae, such as cerebral palsy, hearing impairment, and ocular gaze disorders. In addition, mental impairment and cognitive retardation may occur, seriously affecting the quality of the affected children's life[15]. Therefore, elevated serum total bilirubin levels *in vivo* can be considered as predictors of ABO-HDN, and can also be used for prognostic evaluation and complication monitoring of ABO-HDN. After analyzing the content

of total bilirubin in neonatal serum, it was found that the content of total bilirubin in the serum of neonates with positive hemolysis test tended to increase rapidly in a short time compared with neonates with negative hemolysis test, and the content of total bilirubin in newborns increased significantly when all immunohematological tests were positive. The positive detection rate of ABO-HDN in the three items of hemolysis of the newborn combined with serum total bilirubin, hemoglobin, and Ret% was significantly higher (198 cases, 39.36%) than that in three items of hemolysis alone (156 cases, 31.01%). However, Yin *et al*. pointed out that the detection of serum total bilirubin level was an independent factor for the diagnosis of ABO-HDN after multiple analyses[16]. Although several papers have pointed out that immunohematological tests of hemolysis of newborn combined with total bilirubin detection can significantly improve the detection rate of ABO-HDN compared with immunohematological tests of hemolysis of newborn alone, the relevant mechanism of combined detection has not been fully clarified, and remains to be further studied.

ABO-HDN also causes anemia in newborns, leading to a decrease in hemoglobin and an increase in reticulocyte numbers. In this study, we found that the mean hemoglobin content in newborns showing a positive hemolysis test was significantly lower than that in newborns with a negative hemolysis test. Xue Song *et al*. showed that serum total bilirubin and Ret% values were significantly increased in ABO-HDN positive newborns compared with ABO-HDN negative newborns, and hemoglobin was significantly decreased compared with ABO-HDN negative newborns[17]. Chen *et al*. reported that ABO-HDN could cause liver and kidney injury and even myocardial injury in newborns[18]. Therefore, in pediatric clinical work, medical workers should not only pay attention to the clinical manifestations of ABO-HDN newborns such as severe jaundice leading to kernicterus and late neurological dysfunction, but also be vigilant for other system and organ damage, in order to achieve timely detection and treatment.

In summary, the antibody elution test had the highest sensitivity for neonatal ABO-HDN, confirming that serum total bilirubin levels and Ret% were significantly increased, while hemoglobin levels were significantly decreased in ABO-HDN-positive neonates. In addition, serum total bilirubin, Hb, and Ret% detection combined with immunohematological tests of neonatal hemolysis can effectively improve the detection rate of ABO-HDN. Combined serologic and immunohematological tests can facilitate the early diagnosis and treatment of HDN in newborns.

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