



BASIC SCIENCE ARTICLE

Prediction of ABO hemolytic disease of the newborn using pre- and perinatal quantification of maternal anti-A/anti-B IgG titer

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BACKGROUND: Hemolysis in fetus/newborns is often caused by maternal antibodies. There are currently no established screening procedures for maternal ABO antibodies harmful to fetus/newborn. We investigated the clinical significance, and predictive value of maternal anti-A/B titer for hyperbilirubinemia in ABO-incompatible newborns.

METHODS: We conducted a case–control study of blood group O mothers and their ABO-compatible (O) vs. -incompatible (A/B) newborns receiving phototherapy, and of ABO-incompatible newborns receiving phototherapy vs. no phototherapy. Newborn data and treatment modalities were recorded, and total serum bilirubin and hemoglobin were measured. Maternal anti-A/B immunoglobulin- γ (IgG) titers were measured prenatally and perinatally, and negative and positive predictive values (NPV, PPV) were calculated to assess the risk of developing hyperbilirubinemia requiring phototherapy.

RESULTS: We found a significantly higher maternal IgG antibody titer in the case group ($p < 0.001$). Maternal anti-A/B titers at first trimester had modest predictive values: NPV = 0.82 and PPV = 0.65 for neonatal hyperbilirubinemia; titers at birth improved the predictive values: NPV = 0.93 and PPV = 0.73. Newborn hemoglobin was significantly lower in incompatibles compared to compatibles ($p = 0.034$). Furthermore, increased anti-A/B IgG production during pregnancy was associated with hyperbilirubinemia and hemolysis in incompatible newborns.

CONCLUSIONS: There was a significant association between maternal anti-A/B IgG titer and hyperbilirubinemia requiring treatment.

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IMPACT:

- Maternal anti-A/B IgG titer in the first trimester and at birth is predictive of hemolytic disease of the ABO-incompatible newborn.
- Increased IgG anti-A/B production throughout pregnancy in mothers to ABO-incompatible newborns developing hyperbilirubinemia contrasts a constant or reduced production in mothers to newborns not developing hyperbilirubinemia.
- Screening tools available in most immunohematology laboratories can identify clinically important IgG anti-A/B.
- Use of maternal samples taken at birth yielded NPV = 0.93 and PPV = 0.73.

INTRODUCTION

Newborn jaundice is a very common condition appearing after the first 1–2 days of life in >60% of term newborns. This jaundice is caused by unconjugated hyperbilirubinemia accumulating in the organism, and it is due to the temporarily immature metabolic pathways of the liver. The condition is often benign and self-limiting. However, if the unconjugated hyperbilirubinemia rises to high levels, it can be toxic to the central nervous system and cause acute bilirubin encephalopathy (ABE) and kernicterus spectrum

disorder (KSD).¹ Extreme levels of bilirubin in newborns are often caused by hemolysis, resulting in an early increased production of bilirubin at a time when liver metabolism is still immature. Phototherapy is effective and is the current treatment of choice for neonatal hyperbilirubinemia. In Scandinavia, 2–3% of all newborns born at term or late preterm are treated with phototherapy.² If phototherapy is started before the level of bilirubin becomes potentially toxic, then invasive treatment such as exchange transfusions can be avoided.²

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Hemolysis in newborns is often caused by maternal antibodies.³ Maternal blood group alloantibodies of the immunoglobulin-y (IgG) class, reactive against fetal red blood cell (RBC) antigens inherited from the father, can be transferred across the placenta and destroy fetal cells via the phagocytosis system of the fetus and newborn causing hemolytic disease in the fetus and newborn (HDFN). The success of RhD immunoprophylaxis has led to a dramatically reduced incidence of RhD (formerly known as Rhesus D) alloimmunization. ABO incompatibility is now the most prevalent cause of HDFN.³ The disease affects newborns with blood group A and B born to mothers with blood group O. ABO incompatibility between mother and newborn appears in 20% of all pregnancies, while 1–4% develops HDFN.⁴ The prevalence is higher among non-Caucasians (3–5%) than among Caucasians (<1%) for uncertain reasons.³ A study of neonatal hyperbilirubinemia in Denmark have reported ABO incompatibility in 15 of 21 cases with total serum bilirubin (TsB) $\geq 600 \mu\text{mol/L}$, which was connected with a substantial risk of KSD; the ethnicity was non-Caucasian in 4 of these cases.⁵

ABO antibodies differ from other clinically important blood group antibodies because they are naturally occurring and largely do not cross the placenta because they are IgM. In the past, serological methodology required cumbersome procedures to distinguish IgG from IgM antibodies and resulted in uncertain analytical results.⁶ Current methods include assays that detect only the IgG class of blood group antibodies, which provide a new opportunity to design a precise screening tool to detect only the clinically important IgG anti-A/B.

There are currently no established screening procedures for maternal ABO antibodies harmful to the fetus and newborn,^{7–9} and in Denmark there is no systematic approach to newborns with jaundice. Measurement of bilirubin is often based on a clinical evaluation and since ~30% of newborns are discharged from the maternity ward after only a few hours, the parents have the major responsibility for the observation of jaundice. However, visual observation for jaundice can be difficult, especially in non-Caucasian newborns.²

In Denmark, as in many other countries, blood samples from all pregnant women between 6 and 12 weeks of gestation are routinely analyzed for the ABO and RhD blood group and screened for irregular blood group antibodies and infection markers. These analyses could be expanded with an additional screening for harmful ABO antibodies to identify a risk of postnatal extreme hyperbilirubinemia.

The aims of this study were, (1) to investigate if anti-A/B IgG titers of blood group O mothers determined with first trimester blood samples are usable for routine screening to predict the risk of hyperbilirubinemia in the newborn caused by ABO-HDFN and (2) to assess the clinical significance and predictive value of the maternal anti-A/B titer at birth and to examine the impact in relation to hyperbilirubinemia.

METHODS

Participants and blood samples

This was a prospective case–control study conducted between August 1, 2017 and December 1, 2018 as a collaborative study between the Department of Clinical Immunology, Rigshospitalet, and the obstetric/neonatal departments at Rigshospitalet, Herlev Hospital, Hvidovre Hospital, and Hillerød Hospital in the Capital Region of Denmark as well as at Aalborg University Hospital in The North Denmark Region. The inclusion criteria were newborns with a gestational age ≥ 35 weeks and blood group O mothers.

From April 1 to December 1, 2018 three groups of newborns and their blood group O mothers were included. (1) Cases ($n = 38$) were blood group A/B-incompatible newborns receiving phototherapy due to hyperbilirubinemia. Two cases were withdrawn from initial 40 cases—one because of a sample mix-up and one

due to signs of large amounts of cryoglobulin potentially interfering with the analyses. (2) Compatibles ($n = 30$) consisted of compatible blood group O newborns receiving phototherapy due to hyperbilirubinemia. This group was only used for comparison with the case group. (3) Controls ($n = 38$) were blood group A/B-incompatible newborns who did not receive phototherapy.

The indication for phototherapy followed the existing guidelines in Denmark. These guidelines are closely related to the Norwegian guidelines from 2011 and have a treatment threshold for phototherapy that gradually increases in the first 3 days of life, reaching a plateau for mature infants $\geq \text{GA } 37 + 0$ weeks and $\geq 2500 \text{ g}$ of TsB of $350 \mu\text{mol/L}$.¹⁰ When TsB reaches $50 \mu\text{mol/L}$ above the treatment threshold for phototherapy, it is recommended to increase the intensity of phototherapy (double/triple) by using phototherapy from multiple sides or decrease the distance from phototherapy device to infant. The attending physician enrolled the participants for the case and compatible group at hospitals in the Capital and Northern Region of Denmark. The control group was enrolled either in connection with the blood sampling for the neonatal screening test (Northern Region) or before discharge from the maternity ward (Capital Region).

After inclusion, from newborns, a 1 mL blood sample was collected in an EDTA-containing tube. The ABO blood grouping was done on receipt of the blood sample. The mothers donated a 6 mL EDTA-stabilized blood sample, which was separated into plasma and a cell-rich suspension and stored below -20°C for later use.

From August 1, 2017 to March 1, 2018 residual plasma from first trimester samples were collected from all blood group O pregnant women at the Department of Clinical Immunology of the Capital Region. Samples were stored below -20°C in a first trimester Biobank. Plasma samples from included mothers were selected from this Biobank using the civil registration number.

Measurements

ABO blood grouping on newborns was done by standard serological methods.¹¹ The maternal plasma samples from the first trimester and at birth were paired, and the anti-A and anti-B IgG titers were analyzed in parallel. Reagent RBCs were in-house single donation glycerol frozen-thawed heterozygous A1 and heterozygous B RBCs suspended in CellStab (REF: B005652, DiaMed, Cressier, Switzerland). The entire process was fully automated using a solid-phase red cell adherence assay (SPRCA) in Capture-R Select microplates (REF: 0006446, Immucor Inc., Norcross, GA, USA) performed on the Galileo Neo analyzer (Immucor Inc.) according to the manufacturer's instructions and with an intralaboratory variation of plus or minus one titer step and a percent coefficient of variation (CV%) of 5.8.¹² Briefly, the wells of the Capture-R Select microplates were coated with a monolayer of either blood group A1 or B RBCs. Serial dilutions of the maternal plasma samples were prepared in phosphate-buffered saline to the following dilutions: 1:8, 1:32, 1:64, 1:128, 1:256, 1:512, 1:1024, and 1:2048. From each dilution, 50 μL was tested in 100 μL low ionic strength solution (REF: 0006420, Immucor Inc.); plasma to cell ratio was 63:1. Visualization of the reaction used anti-IgG-coated indicator RBCs (REF: 0006428, Immucor Inc.). Results were read and interpreted automatically from a captured image by the Immucor software and transformed to agglutination strength ranging from negative to positive (from 1+ to 4+).

For maternal birth samples, a second method was included using a manual column agglutination technology (CAT) method.⁴ The LISS/Coombs card containing anti-IgG (DiaMed, ID-cards no. 50540, Cressier, Switzerland) were used. The same A1 and B RhD-negative RBCs, the same plasma to cell ratio, and the same series of dilutions of plasma were used, although diluted manually with

Table 1. Demographic and clinical data for the newborns.

	Phototherapy		No phototherapy controls	P value, bold <0.05	
	Compatible	Cases		Compatible vs. cases	Cases vs. controls
Newborns, <i>n</i>	30	38	38		
Blood group	O	A or B	A or B		
Blood group B, <i>n</i> (%)					
All newborns		15 (40%)	8 (21%)		0.08
Only Caucasians		11 (39%)	7 (19%)		0.08
Caucasian/non-Caucasian, <i>n</i>	23/5 (NA = 2)	28/9 (NA = 1)	36/2	0.53	0.020
Sex, males, <i>n</i> (%)	18 (60%)	19 (50%)	18 (47%)	0.41	0.49
Birth weight, g ^a	3360 (1450–4200)	3515 (2575–4685)	3665 (2684–4960)	0.35	0.19
Cephalhematoma	1	3		ND	ND
Diabetes mellitus matris	1	1		ND	ND
Gestational diabetes	3	2		ND	ND
Age					
Gestational age, week	38 (35–44)	40 (35–42)	40 (37–42)	0.017	0.40
Postnatal age, h ^{a,b,c}	65 (26.3–267)	50 (5.4–225)		0.006	
Hemoglobin, mmol/L ^{a,b}	11.5 (9.5–15.2)	11.0 (6.6–13.7)		0.034	
TsB ₀ , μmol/L ^{a,b}	291 (128–449)	282 (105–608)		0.62	
TsB _{Max} , μmol/L ^{a,b}	306 (128–449)	295 (140–608)		0.67	
Acute bilirubin encephalopathy ^b					
Symptoms, <i>n</i>	12	7			
Early ^d , <i>n</i>	12	7		0.039	
Intermediate ^e , <i>n</i>	0	0		ND	
Advanced ^f , <i>n</i>	0	0		ND	
Treatment					
Phototherapy ^b					
All newborns				<0.001	
Single, <i>n</i>	28	18			
Double, <i>n</i>	2	12			
Triple, <i>n</i>	0	8			
By ethnicity ^g				0.33/0.11 ^h	
Single, <i>n</i>	22/4 (NA = 2)	16/2			
Double, <i>n</i>	1/1	6/5 (NA = 1)			
Triple, <i>n</i>	0/0	6/2			
IVIg, <i>n</i> ^b	0	5		ND	
Exchange transfusion ^b	0	0		ND	

NA not available, ND not done due to small sample size.

^aMedian (range).

^bOnly measured and registered in the groups receiving phototherapy.

^cPostnatal age when phototherapy was started.

^dLethargia, reduced ability to suck, hypotonia, irritable, jittering, and weak Moro.

^eEarly signs plus hypertonia, opisthotonos, retrocollis, and high-pitched cry.

^fIntermediate signs plus seizures, apnea, sun-setting eyes, pronator spasm of upper extremities, inability to feed, and coma.

^gCaucasians/non-Caucasians.

^hDifferences between Caucasians and non-Caucasians within the compatible and the case group.

an intralaboratory variation of plus or minus one titer step and a CV% of 12.4. The antibody titer was defined as the inverse of the highest dilution with a result of at least 1+ for SPRCA and at least w+ for CAT.

An antibody screening test was also performed by indirect antiglobulin technique and CAT on maternal plasma samples to exclude the presence of other blood group antibodies responsible for HDFN.

Relevant newborn data such as ethnicity, sex, birth weight, cephalohematoma, signs of early to advanced ABE, gestational age, and postnatal age, as well as maternal diabetes mellitus or gestational diabetes, were recorded. Additional treatment modalities, including phototherapy, intravenous immunoglobulin treatment, and exchange transfusion, were recorded. TsB and hemoglobin (Hb) were measured with standard methods by the biochemical department serving the including hospital.

Table 2. Maternal IgG antibody titer levels by the SPRCA.

	Phototherapy		No phototherapy control	P value, bold <0.05 ^a	
	Compatible	Case		Case vs. compatible	Case vs. control
Newborn blood group	O	A or B	A or B		
Maternal antibody	Mean of anti-A and anti-B	Anti-A or anti-B ^b	Anti-A or anti-B ^b		
First trimester ^c , n	17	18	29		
Geometric mean (range)	80 (11–362)	313 (128–2048)	89 (8–1024)	<0.001	<0.001
At birth, n	30	38	38		
Geometric mean (range)	46 (6–256)	313 (8–2048)	64 (4–1024)	<0.001	<0.001
P value, bold <0.05 ^d	0.003	0.942	<0.001		

SPRCA solid-phase red cell adherence assay.

^aIndependent-samples Mann–Whitney U test.

^bThe ABO antibody corresponding to the ABO blood group of the infant.

^cGestational week 6–10.

^dRelated samples Wilcoxon signed-rank test.

Hb was measured at the same time as the initial TsB (TsB₀). This was the measurement guiding phototherapy.

Data protection and ethics

The study was approved by Danish Data Protection Agency, RH-2017-295 and the Regional Scientific Ethical Committee of the Capital region of Denmark (H-17029655). Verbal and written consent was obtained from the mothers and the parents of the newborns.

Statistical analyses

Differences between baseline demographics and clinical variables, as well as antibody titers, were examined within two comparison groups: (1) cases vs. compatibles and (2) cases vs. controls.

χ^2 Test or Fisher's exact test (if expected counts are <5) were used for categorical variables. Mann–Whitney U tests were used to compare unpaired continuous variables, and a Wilcoxon signed-rank test was used for paired continuous variables. Because antibody titers have an exponential development, titers were transformed into logarithmic scales for the statistical analyses and geometric means were used in tables.¹³ All tests were two-sided, and p values ≤ 0.05 were considered statistically significant. Ethnicity was included in the data to underscore its importance in our sample. Sensitivity and specificity for predicting hyperbilirubinemia from the titer values were analyzed by a receiver-operating characteristic (ROC) curve, and the Youden index was calculated.¹⁴ To investigate the relationship between TsB increase per hour and anti-A/B titer, we estimated the increase rate as a proxy by dividing TsB₀ by the postnatal age; here, TsB = 0 at birth was used. The curve used for illustration was made with Microsoft Excel 2016. The negative predictive value (NPV) and positive predictive value (PPV) of the titers produced by the two methods were calculated. Statistical analyses were performed using IBM SPSS statistics 22.

RESULTS

Baseline demographic and clinical data of the 106 newborns are shown in Table 1. In the phototherapy group, the data from the case and the compatible group were comparable regarding ethnicity, sex, birth weight, TsB₀, and maximal TsB (TsB_{max}). However, gestational age was significantly lower in the compatible group ($p = 0.017$), whereas postnatal age at the time of phototherapy initiation, and the concentration of Hb, was significantly lower in the case group ($p = 0.006$ and $p = 0.034$, respectively) (Table 1).

A significantly higher number of newborns were diagnosed with symptoms of ABE in the compatible group ($p = 0.039$), but a significantly higher number of newborns in the case group were treated with more aggressive phototherapy ($p < 0.001$). More non-Caucasians than Caucasians received more aggressive phototherapy in the case group compared to controls (7/9 vs. 12/28), however, without achieving significance ($p = 0.11$).

Cases and controls in the group of newborns with blood group A or B were comparable with respect to demographic and clinical data. However, the frequency of blood group B newborns was higher in the case group without achieving significance ($p = 0.08$) and a significantly higher number of non-Caucasians were also found in the case group ($p = 0.02$) (Table 1). Stratification by ethnicity resulted in a very small adjustment in the frequency of blood group B (40% vs. 39%).

Table 2 shows a significantly higher maternal IgG antibody titer in the case group than in the compatible group and in the control group both at the first trimester and at birth. There was a significant difference between maternal anti-A/B titers at the first trimester and at birth in the compatible and in the control group, but not in the case group (Table 2). We found that maternal anti-A/B titers in both the compatible and the control group decreased between the first trimester and at birth with a comparable titer reduction of 43% and 27%, respectively ($p = 0.22$).

A linear relationship was found between the maternal IgG titer at birth and the TsB increase in $\mu\text{mol/L/h}$ ($p = 0.007$), but the correlation coefficient was quite low ($R^2 = 0.105$) (Fig. 1).

Table 3 compares the routine methods for antibody titration. A significant difference was found between antibody titers of the two methods for both cases and controls ($p < 0.001$). Sensitivity and specificity varied slightly between methods. Potential antibody cut-off titers with corresponding sensitivity, specificity, and Youden indexes at both first trimester and at birth are also shown in Table 3. Choosing cut-off titers by the Youden index (J) for the three different setups result in: a first trimester SPRCA titer cut-off value of 128 ($J = 0.52$) with a sensitivity of 100% and a specificity of 52%; a SPRCA titer at birth of 128 ($J = 0.58$) with a sensitivity of 95% and a specificity of 66%; and a CAT titer at birth of 256 ($J = 0.58$) with a sensitivity of 84% and a specificity of 74%. These cut-off values favor sensitivity to specificity for all three setups. At the first trimester SPRCA, NPV is 0.82 and PPV is 0.65; at birth SPRCA, NPV is 0.93 and PPV is 0.73; and at birth CAT, NPV is 0.83 and PPV is 0.76. Figure 2 shows the ROC curves for all three setups.

No maternal irregular blood group antibodies were found in the antibody screening test.

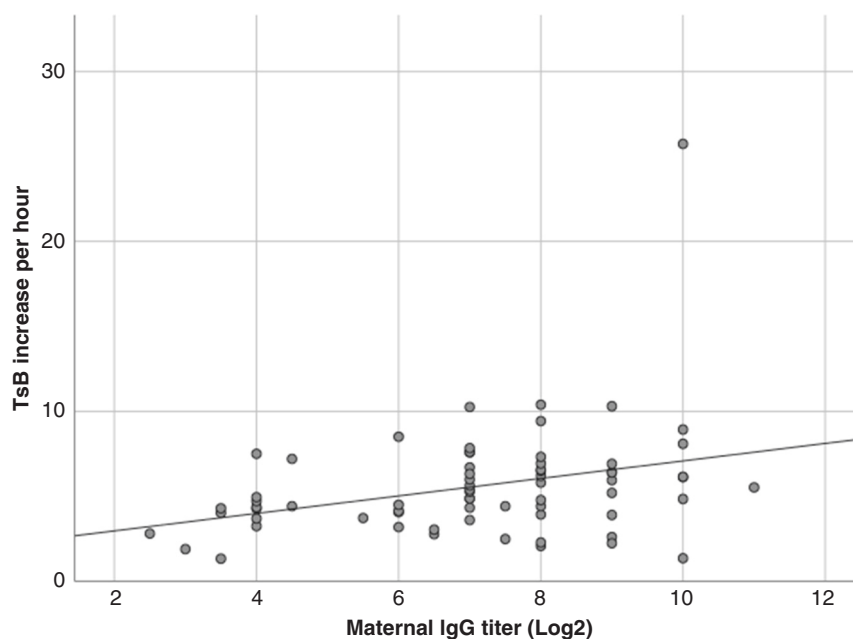


Fig. 1 TsB increase in relation to maternal anti-A/B titers. Scatter plots of maternal IgG titer at birth (Log₂) in relation to TsB increase in μmol/L/h (defined by TsB₀ divided by the postnatal age). R² linear = 0.105.

DISCUSSION

We investigated clinical prediction of hyperbilirubinemia using measurement of the anti-A/B titer in blood group O mothers.

First, the potential of first trimester screening for newborns at risk of ABO-HDFN was investigated by measuring anti-A/B titers of blood group O mothers. Maternal IgG titers were significantly higher in the case group. In our cohort, we found a sensitivity of 100% and a modest specificity of 52% with the SPRCA method for prediction of hyperbilirubinemia treatment. In a population where ABO incompatibility between mother and newborn appears in 20% of pregnancies, our data showed that first trimester screening would give a high number of false-positive screening results probably leading to unnecessary blood tests from the newborn infants. However, prenatal screening may assist in timely action in rare cases of hemolysis in the fetus caused by ABO antibodies.^{15–17}

Second, we measured the maternal anti-A/B titer at birth for comparison. Maternal IgG titers at birth were significantly higher in the case group. For the CAT method, a titer of 256 was determined as the cut-off for developing hyperbilirubinemia that required treatment. The sensitivity and specificity corresponding to that cut-off were 84% and 74%, respectively. This finding is consistent with Bakkeheim et al.⁴ despite their slightly higher cut-off titer of 512, which can be explained by using different A and B reagent RBCs. The cut-off titer at birth determined by SPRCA (128) had slightly different sensitivity and specificity values: 92% and 66%. The clinical value of these measurements should be balanced against both obtaining a new routine blood sample at birth and the measurement of anti-A/B titers—the accuracy of a screening test based on our data might be too low for clinical use. However, hyperbilirubinemia is often multifactorial, and ABO-HDFN may be a contributing cause of more severe hyperbilirubinemia in combination with, for example, glucose-6-phosphate dehydrogenase deficiency or poor feeding.¹⁸ A perinatal screening for newborns at risk of ABO-HDFN would alert the clinician to this added risk factor. A benefit could be that timely phototherapy avoids invasive therapy such as IVIg and assists in the prevention of KSD.

We consider our data interesting from an academic perspective, and to the best of our knowledge, our study is the first to examine the maternal anti-A/B titer in both a first trimester sample and a

perinatal sample. In mothers pregnant with an ABO-incompatible fetus who did not develop hyperbilirubinemia, we found, to our surprise, a decreasing level of maternal anti-A/B IgG between 6 and 10 weeks of gestation and birth. For ABO-compatible pregnancies, essentially the same decline in maternal anti-A/B IgG was observed during the same period of gestation. In the third trimester, maternal plasma volume is physiologically increased by 40%, thus leading to dilution of antibodies.¹⁹ Furthermore, as an important component of the fetal immunological defense against infection, maternal IgG antibodies are transported across the placenta during intrauterine life.²⁰ As a consequence, the total maternal IgG antibody concentration decreases between 9–16 weeks and 37–41 weeks of gestation.²⁰ In contrast, we observed in mothers of the ABO-incompatible fetuses, in whom hyperbilirubinemia developed at birth, that titers remained constant throughout pregnancy. We interpret this as reflecting an accelerated production of antibodies specific for fetal A or B antigens in the case group as opposed to a lack of an increased production in the control group. In other words, ABO-HDFN may not occur only due to the steady-state anti-A/B IgG in blood group O mothers, but may also require an increased maternal IgG production. This response is probably dependent on a specific genetic constitution and a continued exposure to fetal ABO antigens.^{21,22} This suggests that fetal cells or antigens stimulate the maternal immune system. Thus, the first trimester anti-A/B titers by SPRCA had a modest predictive value for pregnancies at risk of the newborn developing HDFN (NPV = 0.82 and PPV = 0.65). Use of samples taken at birth improved the predictive value to NPV = 0.93 and PPV = 0.73. PPV is considered too low for routine screening and we aim to enhance predictive values.

There were more newborns with blood group B and a significantly higher number of non-Caucasians in the case group than in the control group. The results were stratified by ethnicity and this did not explain the higher frequency of newborns with blood group B in the case group. Similar results were found in previous studies, thus corroborating observations that blood group B newborns are more susceptible to ABO-HDFN.^{4,7} Furthermore, the ethnic difference showed a higher frequency of non-Caucasians receiving intensive phototherapy in the form of

Table 3. Comparison of results from the two routine methods, SPRCA and CAT.

	SPRCA	CAT ^a	P value, bold <0.05
Maternal titer at birth^b			
Geometric mean (range)			
Cases (n = 38)	313 (8–2048)	580 (8–8000)	<0.001
Controls (n = 38)	64 (4–1024)	77 (4–2048)	<0.001
First trimester, antibody cut-off titer			
Sensitivity/specificity (Youden index) (n = 18)			
128	1.00/0.52 (0.52)		
256	0.67/0.79 (0.46)		
512	0.33/0.83 (0.16)		
At birth, antibody cut-off titer			
Sensitivity/specificity (Youden index) (n = 38)			
128	0.95/0.66 (0.61)	0.95/0.61 (0.55)	
256	0.74/0.79 (0.53)	0.84/0.74 (0.58)	
512	0.74/0.79 (0.53)	0.66/0.90 (0.55)	

SPRCA solid-phase red cell adherence assay, CAT column agglutination technology.

^aThe ABO antibody corresponding to the ABO blood group of the infant.

^bOnly birth samples were analyzed with the CAT method because these results served as a comparison with previous published data.

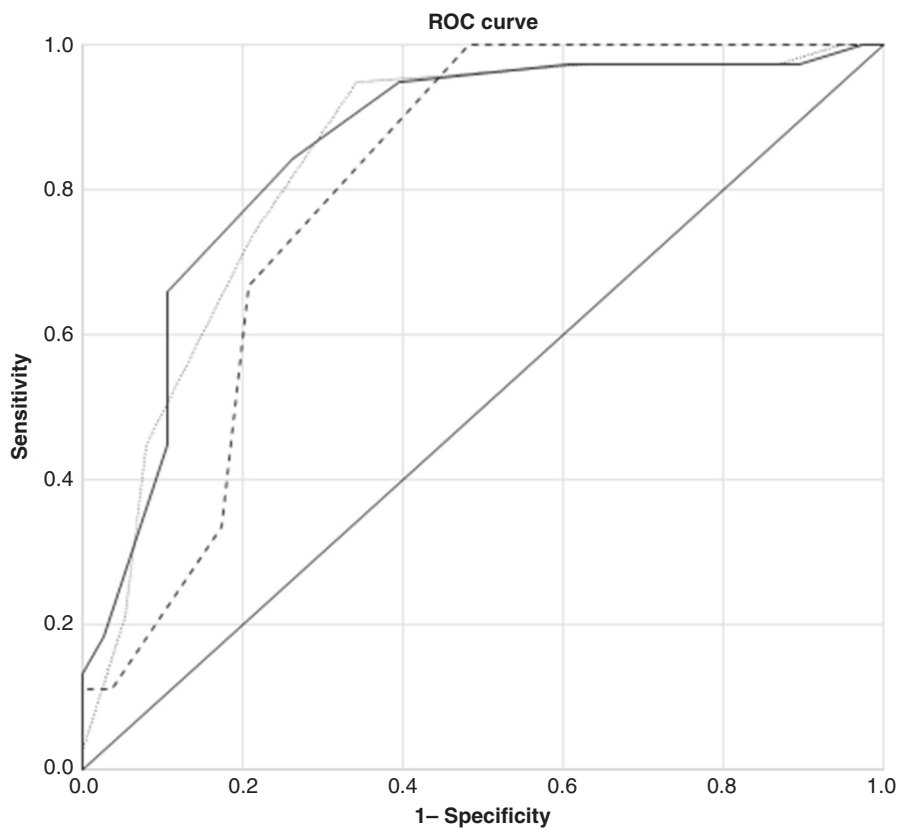


Fig. 2 The diagnostic ability of maternal anti-A/B titers to predict the risk of ABO-HDFN. ROC curve showing results of maternal anti-A/B titers from the first trimester SPRCA (dashed black line) and at birth from both SPRCA (dotted gray line) and CAT (unbroken black line). A reference line (thin black line, $x = y$) is also included. The area under the curve (AUC) indicates that the accuracy of the assay was 0.799 by SPRCA in the first trimester, 0.843 by SPRCA at birth, and 0.854 by CAT at birth with a 95% confidence interval of 0.673–0.924, 0.750–0.935, and 0.765–0.943, respectively.

double light. This could partially be explained by a higher incidence of undiagnosed glucose-6-phosphate dehydrogenase deficiency in the non-Caucasian participants.²³

We included two different methods, SPRCA and CAT, for the determination of maternal IgG anti-A/B titer. We favored the ability to compare our results with findings in a previous study

using CAT and we supplemented with SPRCA to investigate if SPRCA was more accurate in predicting newborns at risk of developing hyperbilirubinemia requiring treatment.⁴ SPRCA has a theoretical advantage by only detecting IgG antibodies, which can cross the placenta, whereas CAT also detects IgM antibodies that react at 37 °C, which are at no risk to the fetus. We found a significant difference in titer between SPRCA and CAT in cases and controls ($p < 0.001$). However, this difference was only 0.5 and 1 dilution step for controls and cases, respectively, and almost equally distributed among all samples. Consequently, CAT had a higher cut-off titer, and the inherent risk of also detecting some IgM anti-A/B did not seem to reduce specificity. We found that maternal anti-A/B with the highest titer was directed to the ABO blood group of the fetus. In practice, a screening for high titers might therefore be done with blood group AB reagent RBCs and ignoring the exact specificity of the ABO antibody.

Our case group differed from the compatible group in several aspects: ABO incompatibility and a high level of maternal anti-A/B was associated with earlier phototherapy and a lower level of Hb, strongly indicating hemolysis. In the compatible group, the lower gestational age could be a predisposition factor for hyperbilirubinemia caused by the more immature metabolic pathways in the liver.¹⁰ Earlier and more aggressive treatment in the case group could explain why the highest level of bilirubin was similar to the compatible group. However, we were surprised that we could not find a stronger association between the level of maternal anti-A/B and the rate of bilirubin increase as previously demonstrated in other alloimmune antibodies like anti-D.²⁴ We speculate that variable expression of the A and B antigens on fetal RBCs or a variable maternal anti-A/B IgG subclass profile could be limitations for screening tests based on maternal anti-A/B. Further studies should investigate these factors to improve the accuracy of predicting newborns at risk of severe hyperbilirubinemia and to identify newborns at high risk of a rapid TsB increase.

The strengths of this study were that maternal IgG titers were semi-quantitated in both a first trimester sample and in a sample taken at birth. We also investigated two different methods for quantitating titers. A limitation of our study was that the number of cases were low on stratified data, for example, newborn blood group and phototherapy intensity. Unfortunately, first trimester samples were not available from all mothers.

CONCLUSION

This study shows a significant association between high maternal anti-A/B IgG titer and the development of hyperbilirubinemia requiring treatment in the newborns, with the titer at birth having the highest predictive values. The findings underline the clinical significance of anti-A/B. Examining the maternal anti-A/B titer in both a first trimester sample and a sample taken at birth revealed an increased IgG anti-A/B production throughout pregnancy in mothers of incompatible newborns developing hyperbilirubinemia.

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AUTHOR CONTRIBUTIONS

G.R.K. conceptualized and designed the study, collected the data, performed the statistical analyses, drafted the initial manuscript, and reviewed and revised the

manuscript. M.L.D. conceptualized and designed the study, included patients, reviewed thoroughly the statistical analyses and clinical aspect, and revised the manuscript. B.M.H. conceptualized and designed the study, included patients, reviewed thoroughly the clinical aspect, and revised the manuscript. H.L. designed tables, reviewed thoroughly the statistical analyses, and reviewed and revised the manuscript. K.V.J., A.K.-T., P.A., and T.B. included patients, and reviewed and revised the manuscript. F.B.C., F.E., M.K.S., and M.H.D. conceptualized and designed the study, and reviewed and revised the manuscript. All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work. This work was supported by grants from Rigshospitalet's and dbio's Research Foundations.

ADDITIONAL INFORMATION

Competing interests: The authors declare no competing interests.

Patient consent: Verbal and written consent was obtained from the mothers and the parents of the newborns.

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