Improving care for red blood cell alloimmunized pregnant women

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Chapter 6

Diagnostic value of laboratory monitoring to predict severe hemolytic disease of the fetus and newborn in non-D and non-K-alloimmunized pregnancies

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Abstract

Background: Pregnant women are routinely screened for red blood cell (RBC) antibodies early in pregnancy. If RBC-alloantibodies are detected, repeated laboratory testing is advised to timely identify pregnancies at high risk for severe hemolytic disease of the fetus and newborn (HDFN). We assessed for RBC alloantibodies, other than anti-D or anti-K, cut-offs for the titer and the antibody dependent cellular cytotoxicity (ADCC) test to select high-risk cases. To advise on test repeat intervals, and to avoid unnecessary testing, we evaluated the chance for exceeding the cut-offs for Rh antibodies other than anti-D, Jk, Fy, and S/s antibodies.

Study design and methods: Diagnostic value of antibody titer and ADCC test was determined with data from a prospective index-cohort study, conducted in 2002-2004. Laboratory test outcomes were from a recent observational cohort (2015-2016).

Results: A titer cut-off of ≥16 showed a sensitivity of 100% (95% CI:73-100%) and a positive predictive value (PPV) of 17% (95% CI:14%-20%). The percentage of pregnancies reaching a titer above the cut-off of ≥16 varied from 0% for anti-Jka / Jk^b (n = 38) to 36% for anti-c (n = 97). The ADCC test showed no cut-off with a 100% sensitivity. However, in cases with a titer ≥16 and an ADCC test ≥30% a PPV of 38% was obtained to detect severe HDFN.

Conclusion: A titer cut-off of ≥16 is adequate to detect all cases at risk for severe HDFN; the ADCC test may add a more accurate risk estimation. Repeated testing is recommended in pregnancies with anti-c. In pregnancies with other Rh antibodies a repeated test in the third trimester is recommended.

Introduction

In most western countries pregnant women are routinely screened early in pregnancy for the presence of red blood cell (RBC) alloantibodies. (25, 26, 64, 158) RBC alloantibodies of the mother can cause hemolytic disease of the fetus and newborn (HDFN). HDFN is characterized by anemia, which can occur early in pregnancy, and by high bilirubin levels after birth. Not all RBC alloantibody specificities cause HDFN. Furthermore, the titer of the RBC alloantibody and its biologic activity are correlated with a mild or more severe course of disease. To timely identify pregnancies at risk for a severe course of HDFN, defined as a need of fetal therapy, preterm delivery, or intensive neonatal treatment, repeated laboratory testing during pregnancy is advised.(25, 26, 64, 158) In this pre-selected group, fetal anemia can be diagnosed with a high sensitivity and specificity by non-invasive ultrasonography, using Doppler middle cerebral artery blood (MCA) flow velocity measurements.(25, 29)

Most cases of severe HDFN are caused by anti-D, less frequently by anti-c and anti-K, and in a rare case by other Rh antibodies.(1-4, 6) Anti-Fya/-Fyb increases the risk for neonatal icterus, needing phototherapy treatment.(6) For almost all other RBC alloantibody specificities there is only casuistic evidence that these cause a severe HDFN disease trajectory.(4, 7, 10)

The policy of laboratory monitoring in alloimmunized pregnant women varies between countries from 4-week intervals to once at 28-34 weeks or only pregnant women with anti-D, –c, and/or -K on a regular basis. When a certain cut-off value (“critical titer”) is exceeded, patients are referred to a maternal-fetal medicine center for close surveillance and, if needed, for fetal or neonatal treatment.(25, 26, 64, 158)

In the Netherlands, in pregnancies with clinically relevant RBC alloantibodies and a fetus (possibly) positive for the cognate antigen, not only serial antibody titer measurements are advised, but also a monocyte-driven antibody-dependent cellular cytotoxicity (ADCC) assay to determine the destructive capacity of the antibodies.4 In case of anti-D, the ADCC is validated to discriminate well between pregnancies with low and high risk for severe HDFN.(7, 19, 139, 159)

For anti-K, we recently reported the results of a nationwide study (1999-2015) concluding that for K-immunized pregnancies a critical titer of four should be used to select pregnancies at high risk for fetal hemolysis, while the ADCC test appeared not to add to the selection of cases at risk for severe HDFN.(160)

In general, for non-D/non-K alloantibodies a cut-off level of 32 is used (reviewed by Moise et al.)(4, 159) and confirmed by a study of Hackney et al., indicating that all cases of severe HDFN with need for IUT or with neonatal hemoglobin levels <10 g/

dL were identified with a titer cut-off of 32. (59) Similarly, Joy et al. concluded for anti-E a titer cut-off value of 32.(161)

In the Netherlands, the titer cut-off value of ≥16 and/or an ADCC test result of ≥30% is used to timely select pregnancies at risk for fetal hemolysis by non-D/non-K alloantibodies. We present the data underlying this policy.(26)

The main goal of laboratory testing is to timely identify all pregnancies possibly at risk for fetal hemolysis, with an almost 100% sensitivity. This might result in a high proportion of unnecessary tests and unnecessary health care costs. For pregnant women, frequent laboratory testing is invasive and can cause unnecessary anxiety; on the other hand, it can also be reassuring.

The aim of the current study was to gain more insight into the optimal frequency of laboratory testing in pregnancies complicated by RBC alloantibodies other than anti-D or anti-K. Therefore, we first report the evidence underpinning the previously determined cut-offs for titer and ADCC test in pregnancies complicated by non-D/non-K RBC alloantibodies, in order to detect severe HDFN requiring transfusion therapy. Second, to assess the added value of repeated laboratory testing for selection of high-risk cases, we investigated the chance of exceeding the determined cut-offs, according to antibody specificity.

Materials and methods

Organization of the prevention program in the Netherlands

In the Netherlands, all pregnant women are typed for ABO, D and c blood group antigens, and screened for RBC antibodies at the first trimester booking visit. All screen-positive samples are sent to one of two national reference laboratories for confirmation and determination of the antibody specificity: Sanquin Diagnostics, Amsterdam (90% of the pregnant population) and the Special Institute for Blood Group Investigations (BIBO), Groningen (10% of the pregnant population). When RBC antibodies are detected with the potency to destroy fetal RBC's, the father of the fetus is typed for the cognate antigen(s). If the father is heterozygous or if his antigen type is unknown, non-invasive fetal typing with cell-free fetal DNA isolated from maternal plasma is offered (for RHD, RHC, RHc, RHE, and K), since 2004.(24, 26) If the fetus is (probably) antigen-positive, serial titration of maternal antibodies and the ADCC test are performed.

The two reference laboratories use an expert opinion–based protocol to determine the interval for repeat testing. The interval depends on the antibody specificity

and the test results: pregnancies with anti-D, anti-K, or anti-c are monitored most frequently, with a 2-week interval during the third trimester. Other Rh antibodies (anti-C/-E/-e) are monitored every 3 weeks during the last trimester, while in case of other antibodies (anti-Fya/-Fyb, -Jka/-Jkb, -S/-s and other) the laboratory testing is repeated only once in Week 30. Since 2009, the Dutch guideline states that for antibodies other than anti-D or anti-K, a titer ≥16 and/or an ADCC test result ≥30% indicates a risk for HDFN; in these cases the fetus will be monitored with MCA Doppler measurements in a specialized center with a frequency depending on the antibody specificity.(58) Laboratory follow-up is usually discontinued if these cut-offs are reached. Severe fetal anemia is treated with IUTs at the Leiden University Medical Center (LUMC), the national Dutch reference center for fetal therapy.

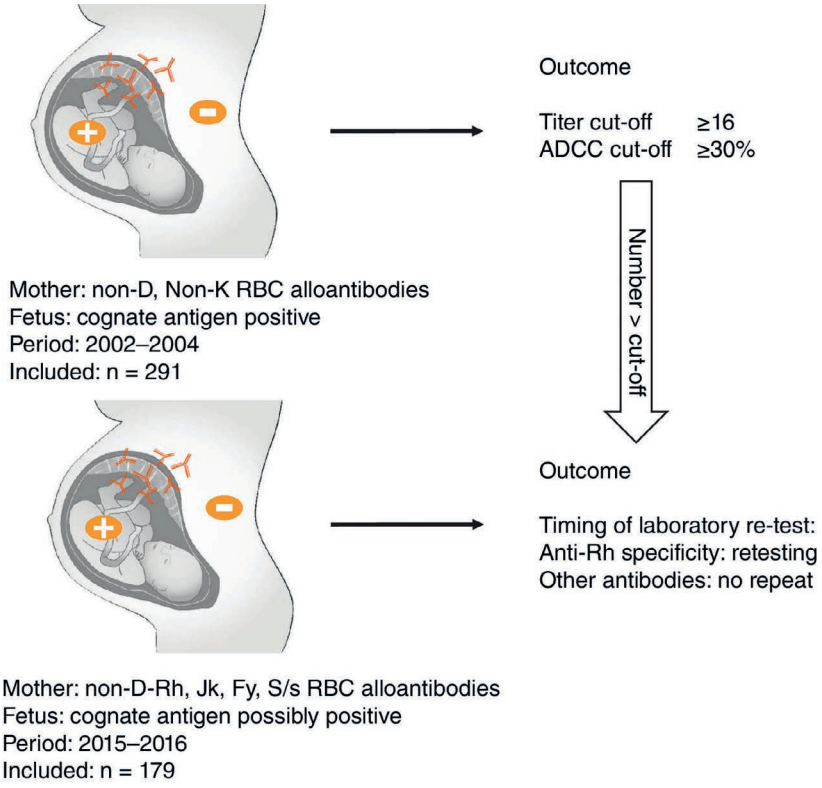
Laboratory testing

Both reference laboratories perform antibody titration in tubes, in phosphate-buffered saline with addition of 2% of a 22% solution of bovine serum albumin, by doubling dilutions, with an incubation time of 30 minutes, with the indirect antiglobulin test (IAGT), using an anti-IgG reagent. For Rh antibodies, (anti-c, -D, and -E), double-dose antigen-positive RBCs were used and for all other antibody specificities (e.g., anti-C, anti-e, -K, -Fya, -Fyb, –Jka, –Jkb, -S, –s) single-dose antigen positive RBCs. Double-dose c, D, and E positive RBCs are also used in the ADCC test. The ADCC test, as described by Engelfriet and Ouwehand, is performed at Sanquin Diagnostics in Amsterdam for all Dutch samples.15 Fetal typing is also only performed at Sanquin Diagnostics.

Study design and study population

An outline of the study is provided in Fig. 1. To assess the diagnostic value of laboratory testing with titer and ADCC test, data were used from a nationwide prospective index cohort study, conducted to evaluate the effectiveness of first trimester RBC antibody screening for early detection of cases at risk for HDFN (OPZI-study). All pregnant women with clinically relevant non-D RBC antibodies, recognized by routine first trimester screening (n = 1,002) from September 1, 2002 until June 1, 2003 and October 1, 2003 until July 1, 2004 (population: n = 306,000) were included. This study is described in more detail, in a previous publication.(10)

Figure 1: *Outline of studies to determine test algorithm for non-D, non-K red blood cell (RBC) alloantibody screening in pregnancy.*



A case was included in the current study if the fetus was positive for one or more antigens against which maternal antibodies were present during pregnancy and if the last test result of laboratory monitoring was performed ≥32nd week of pregnancy or within 21 days before birth, or the last test result indicated a risk for severe HDFN, needing clinical monitoring (titer ≥64 or ADCC test result ≥50%). When antibodies were newly detected in cord blood, for which no laboratory monitoring was performed during pregnancy, the case was excluded.

In a second cohort, we evaluated the results of the current policy of laboratory monitoring. For this purpose, we performed a retrospective study, including all women with Rh antibodies, other than D (anti-c/-C, -E/-e), anti-Fya/-Fyb, anti-Jka/-Jkb and/or anti-S/-s, but without the presence of anti-D and/or anti-K, detected at first trimester antibody screening in 2015 and 2016, at risk for HDFN (partner positive for the cognate antigen and/or positive result of non-invasive fetal typing with cell-free DNA). Cases were selected at Sanquin Diagnostic Services.

Outcomes

The primary outcome of the first part of our study was the diagnostic value of laboratory testing, to predict severe HDFN, defined as the need for antenatal or neonatal transfusion therapy during the first week of life, or mild HDFN (only neonatal phototherapy). Intensive phototherapy, starting immediately after birth to prevent (exchange) transfusions in children at high risk for severe HDFN, was not usual care during the study period.(15) In twins with two antigen positive children, the outcome of the most severely affected child was used to categorize disease severity.

We aimed to evaluate the current policy of laboratory monitoring in a second observational cohort, by assessing the chance for exceeding the laboratory cut-offs.

Data collection

For the study on diagnostic value, information about laboratory testing (antibody specificity, antigen typing of father and fetus, titers, and ADCC test results) was collected at the two reference laboratories that routinely perform these analyses. As part of the OPZI-study, the newborn's antigen typing, RBC alloantibody screen, including analysis of an eluate was determined.(10) Clinical outcome data (i.e., number of IUT(s), neonatal blood transfusion(s), phototherapy, gestational age at birth, perinatal death) and neonatal laboratory test results (DAT, antigen typing, hemoglobin level, bilirubin level) were collected from the obstetric care provider. To evaluate the current policy of laboratory monitoring, in the second part of this study, we collected the laboratory testing results from Sanquin Diagnostics (titers, ADCC test, antigen typing of the father, fetal genotyping, formation of additional antibodies) of all samples obtained during pregnancy.(10) No clinical data were collected. We calculated the gestational age at each follow-up laboratory test from the time interval in days between this follow-up test and the routine first trimester blood sampling during pregnancy, assuming that the first trimester screening was performed at 12 weeks (84 days).

Analysis

Categorical variables were described as number and percentage and continuous variables by median and interquartile range P25%-P75%. Associations between categorical variables were tested by Pearson's chi-square test.

Test characteristics (sensitivity, specificity, positive and negative predictive values) for the prediction of severe HDFN (antenatal or neonatal transfusion therapy) were calculated with 2 × 2 tables for different cut-off levels. To establish the optimal cut-off for antibody titer and ADCC test results, receiver operating characteristic curves (ROCs) were constructed. We considered an area under the ROC curve (AUC) of 0.8 or more as a useful predictive test.

All analyses were performed with SPSS version 24.0, except the confidence intervals for measures of diagnostic value. These were calculated with MedCalc version 18.11, available via https://www.medcalc.org/calc/diagnostic_test.php.

Ethical considerations

The index cohort study was approved by the ethical review board of the Academic Medical Center (AMC) Amsterdam. Consent was given by all women included in the study.

In the retrospective study, the laboratory data were anonymized. The data were stored according to the Dutch established codes of conduct for responsible use of patient material and data, as approved by the Leiden University Medical Center. Ethical approval was not necessary according to the Dutch law on medical scientific research involving human subjects and according to the website of the Central Committee on Research involving Human Subjects.

Results

Study population

The index cohort study performed from 2002 to 2004 included 1,002 women with RBC alloantibodies other than anti-D, of which 900 gave consent for collection of cord blood and clinical data. After exclusion of mothers with children with a negative or unknown antigen typing, antigen-positive children of mothers with anti-K, and of mothers with only one laboratory test or a last testing before 32 weeks of the antibody for which the child was antigen-positive, 291 pregnancies remained for

analysis. (Fig. 1 and Fig. S1, available as supporting information in the online version of this paper.)

In the subsequently performed cohort study covering 2015 and 2016, 516 pregnancies (510 women) with RBC alloantibodies directed against antigens in the Rh system other than D, anti-Fya/-Fyb, anti-Jka/-Jkb and/or anti-S/-s, respectively were included. After exclusion of pregnancies with additional anti-D and/or anti-K, and of pregnancies not at risk for HDFN because the partner was antigen negative for all the cognate antigens or the fetus was antigen negative, 279 pregnancies remained for analysis. (Fig. 1 and Fig. S2, available as supporting information in the online version of this paper.)

Diagnostic value of RBC alloantibody titer and ADCC test

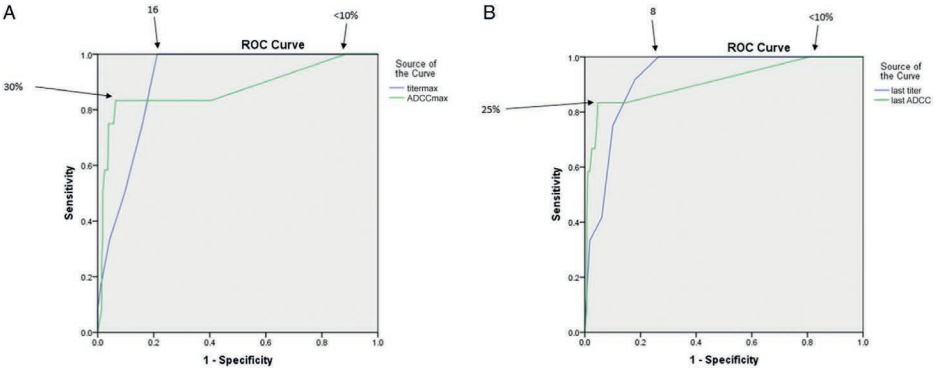
Of the 291 included cases in the index cohort study, 12 children showed severe HDFN, and treatment with an IUT or (exchange) transfusion was needed. This was caused in 10 cases by anti-c or anti-c + anti-E immunization.6 Two other cases were caused by anti-e and by anti-C + anti-Jka, respectively.6 In 49 cases (17%) only phototherapy was given; these cases were mainly caused by anti-c, anti-E, or anti-Fya (Table S1, available as supporting information in the online version of this paper). The ROC to predict the need for antenatal or neonatal transfusion using either the maximum titer, the last titer, or the ADCC test result all showed AUCs above the predefined cut-off of 0.80, with slightly higher values for the last titer and ADCC test result compared to the maximum titer and ADCC. The AUCs to predict the need for transfusion from the first titer or ADCC showed AUCs below the predefined cut-off (Fig. 2A,B and Table 1).

Table 1. *AUC* of maximum and last titer and ADCC test result to predict HDFN disease severity in pregnancies at risk for HDFN*

	Predicted outcome		
	Need antenatal or neonatal transfusionAUC*(95% CI)	Need neonatal phototherapy AUC*(95% CI)	Need antenatal or neonatal transfusion or phototherapy AUC*(95% CI)
Maximum titer	0.90 (0.86-0.95)	0.69 (0.62-0.77)	0.76 (0.69-0.83)
Last titer	0.93 (0.88-0.97)	0.66 (0.58-0.75)	0.74 (0.67-0.81)
Maximum ADCC	0.87 (0.73-1.00)	0.61 (0.52-0.71)	0.68 (0.60-0.77)
Last ADCC	0.91 (0.80-1.00)	0.58 (0.49-0.68)	0.67 (0.58-0.75)

All children were positive for the cognate antigen.
** AUC = Area Under the Curve.*

Figure 2A,B:



A) Receiver Operating Curve to predict the need for transfusion because of HDFN by non-D–/non-K antibodies (n = 291) from the maximum titer and ADCC test result. Legend: All children were positive for the cognate antigen. B) Receiver Operating Curve to predict the need for transfusion because of HDFN by non-D/non-K antibodies (n = 291) from the last titer and ADCC test result. Legend: All children were positive for the cognate antigen.

The AUCs to detect the need for phototherapy and the need for transfusion or phototherapy were all below the predefined cut-off (Table 1, Figs. S3 and S4, available as supporting information in the online version of this paper).

Sensitivity, specificity, and predictive values

To detect HDFN with a need of transfusion therapy, a cut-off for the maximum titer of ≥16 resulted in a sensitivity of 100% with a specificity of 79% and a positive predictive value (PPV) of 17%. In the 10 cases with anti-c, a titer of 16 showed a sensitivity of 100% with a specificity of 69% and a PPV of 27% to detect severe HDFN, needing transfusion therapy (Table 2). For the other RBC alloantibody specificities the PPV was only 6% (Table 2). In the severe HDFN case caused by anti-e, the maximum titer and ADCC test were 32, respectively 70%; in the severe case caused by anti-C + anti-Jka these were 16, respectively <10%.

Table 2. *Number of positive and negative tests, sensitivity, specificity, and predictive values to predict the need for antenatal or neonatal transfusion therapy, by cut-off maximum titer in pregnancies with RBC alloantibodies* and a child positive for the cognate antigen(s)*

n = 291			Need for antenatal or neonatal transfusion therapy (n = 12)				
Cut-off	Test result		True positives	Sensitivity	Specificity	PPV†	NPV†
	+n	−n		% (95% CI)	% (95% CI)	% (95% CI)	% (95% CI)
≥16							
all cases	72	219	12	100 (73.5-100)	78.5 (73.2-83.2)	16.7 (13.8-20.0)	100
anti-c	37	60	10	100 (69.2-100)	69.0 (58.1-78.5)	27.0 (21.3-33.6)	100
other Rh‡	22	89	2	100 (15.8-100)	81.7 (73.1-88.4)	9.1 (6.3-12.9)	100
≥32							
all cases	54	237	9	75.0 (42.8-94.5)	83.9 (79.0-88.0)	16.7 (11.6-23.4)	98.7 (96.7-99.5)
anti-c	27	70	8	80.0 (44.4-97.5)	78.2 (68.0-86.3)	29.6 (20.3-41.1)	97.1 (90.7-99.2)
other Rh‡	17	94	1	50.00 (1.3-98.7)	85.3 (77.3-91.4)	5.9 (1.4-21.2)	98.9 (95.8-99.7)

** RBC alloantibodies = red blood cell alloantibodies; all antibodies, excluding anti-D and anti-K.*

† PPV = positive predictive value; NPV = negative predictive value.

‡ other Rh = all Rh antibodies, excluding anti-D and anti-c.

The ADCC test showed a higher specificity and higher PPVs, but did not reach a 100% sensitivity (Table **S3**, available as supporting information in the online version of this paper). Two cases with the need for transfusion, both with a maximum titer of 16, were missed by using the ADCC test result only. Also no cut-off for the titer or ADCC test with a 100% sensitivity could be determined to detect the need for phototherapy only ór for transfusion and phototherapy combined.

Added value of ADCC test

The added value of the ADCC test to detect severe HDFN was investigated in the 72 cases with a titer ≥16. The ROC-curve showed an AUC of 0.774 (95% CI 0.597-0.950). The PPV of the ADCC test in this group increased from 17.5% to 60% with an ADCC test result of respectively ≥10% and ≥ 60%. In the presence of anti-c the PPV increased from 29% to 83%, in the presence of other antibodies from 4% to 25% (Table **3**).

Table 3. *Number of positive and negative tests, sensitivity, specificity, and predictive values to predict the need for antenatal or neonatal transfusion therapy, by cut-off maximum ADCC test in pregnancies with RBC alloantibodies* and a titer ≥16 and a child positive for the cognate antigen.*

N = 72			Need for antenatal or neonatal transfusion therapy (n = 12)				
Cut-off	**Test result**			**Sensitivity**	**Specificity**	**PPV†**	**NPV†**
	+n	**-n**	**True positives**	**% (95% CI)**	**% (95% CI)**	**% (95% CI)**	**% (95% CI)**
≥10%							
All cases	57	15	10	83.3 (51.6-97.9)	21.7 (12.1-34.2)	17.5 (13.8-22.1)	86.7 (62.7-96.2)
anti-c	31	6	9	90.0 (55.5-99.8)	18.5 (6.3-38.1)	29.0 (23.7-35.0)	83.3 (39.9-97.4)
Rh other‡	15	7	1	50.00 (1.3-98.7)	30.0 (11.9-54.3)	6.7 (1.7-22.7)	85.7 (56.3-96.6)
≥30%							
All cases	26	46	10	83.3 (51.6-97.9)	73.3 (60.3-83.9)	38.5 (27.7-50.5)	95.7 (86.0-98.7)
anti-c	19	18	9	90.0 (55.5-99.8)	63.0 (42.4-80.6)	47.4 (34.6-60.5)	94.4 (72.1-99.1)
Rh other‡	2	20	1	50.0 (1.3-98.7)	95.0 (75.1-99.9)	50.0 (8.6-91.4)	95.0 (82.6-98.7)
≥60%							
All cases	10	62	6	50.0 (21.1-78.9)	93.3 (83.8-98.2)	60.0 (33.2-81.9)	90.3 (84.1-94.3)
anti-c	6	31	5	50.0 (18.7-81.3)	96.3 (81.0-99.9)	83.3 (39.9-97.4)	83.9 (73.6-90.7)
Rh other‡	2	20	1	50.0 (1.3-98.7)	95.0 (75.1-99.9)	50.0 (8.6-91.4)	95.0 (82.6-98.7)

** RBC alloantibodies = red blood cell alloantibodies; all antibodies, excluding anti-D and anti-K.*

† PPV = positive predictive value; NPV = negative predictive value.

‡ other Rh = all Rh antibodies, excluding anti-D and anti-c.

Chance of exceeding the cut-offs

In the observational cohort comprising the years 2002 to 2004, the median number of laboratory tests was 6 (range 1-12) for all RBC alloantibody specificities. In this cohort, including only antigen-positive children, 25% of cases reached a titer of ≥16: anti-c 38%, anti-E 23%, other Rh antibodies 12.5%, anti-Fy^{a}/-Fyb 25%, other antibodies 8.5%. Here it should be noted that anti-c and anti-E are both tested with double-dose antigen positive cells.

From the cohort tested in 2015 and 2016 no follow-up samples were received in 23 out of 279 pregnancies (8%), despite a titer <16 and an ADCC test result <30% and the advice to repeat laboratory testing. This may be because of a miscarriage or because the father was typed antigen negative in the referring center. In the remaining 256 pregnancies, the median number of tests was four (range 1-13), varying from three tests if anti-Fy^{a}/-Fy^{b}, anti-Jk^{a}/-Jk^{b}, or anti-S/-s were present to 6.5 if Rh alloantibodies (other than anti-D) were present.

In 11 cases (4%), the first test results were already above the set cut-offs: anti-c n = 7; anti-c + anti-E n = 1, anti-E n = 3. In another 35 cases the test cut offs for the titer were exceeded during follow up. The risk for exceeding the cut-off differed significantly between antibody specificities, with the highest risk (36%) for anti-c. In none of the pregnancies with anti-C, anti-e, anti-Jk^{a}, and anti-Jk^{b}, the cut-offs were exceeded. The most frequent additional antibody, developed during pregnancy, was anti-c, in addition to anti-E (Table 4).

Table 4. *Number of cases with a titer ≥16 and development of additional RBC alloantibodies**

Antibody specificity N=256	n	n	>Cut-off n (%)	n	Additional antibodies, developed during pregnancy (n = 15)
Anti-c	73		26 (36)		
anti-c		51		21	1 × anti-Fy^a
anti-c + anti-E		18		4	1 × C^w
anti-c + anti-E + anti-Jk^a		1		0	
anti-c + anti-Jk^a		2		0	
anti-c + anti-Kp^a + anti-Wr^a		1		1	
Anti-E	78		15 (19)		
anti-E		77		15	1 × anti-D, 6 × anti-c
anti-E + anti-Jk^a		1		0	
Anti-C/anti-e	14		0		
anti-C		5		0	1 × anti-Jk^a
anti-e		6		0	1 × anti-C
anti-C + anti-e/anti-Ce		2		0	
anti-e + anti-Fy^b		1		0	
Anti-Fy^a/anti-Fy^b	24		3 (12)		
anti-Fy^a		19		3	1 × anti-C
anti-Fy^b		3		0	
anti-Fy^a + anti-S		1		0	
anti-Fy^a + anti-f		1		0	1 × anti-Jk^b
Anti-Jk^a/anti-Jk^b	38		0		
anti-Jk^a		36			1 × anti-f
anti-Jk^a + anti-S		1			
anti-Jk^a + anti-C^w		1			
Anti-S/anti-s	29		2 (7)		
anti-S		27		1	
anti-s		2		1	1 × anti-E
Total	256		46 (18		9 (4)
p value†			<0.001		

** Included are all pregnancies with anti-c/-C,-E/-e, Duffy antibodies, Kidd antibodies, and/or S/s antibodies, but without D and/or K antibodies, detected at first trimester antibody screening in 2015 and 2016 in the Netherlands, at risk for HDFN (partner positive for the cognate antigen and/or positive result of non-invasive fetal typing with cell-free DNA). † p value of testing differences between groups of antibodies (anti-c, -E, -C/-e, -Fya,b, Jka,b, -S/s) in the risk for exceeding the cut-off <0.001 (Pearson's chi square = 29.798, 5 df).*

Discussion

In a nationwide prospective cohort study, including pregnant women with RBC alloantibodies with a specificity other than anti-D or anti-K and an antigen-positive fetus, we found that the maximum titer was, compared to the highest titer and the ADCC test, the best test to differentiate between pregnancies at low and high risk for severe HDFN. A cut-off of ≥16 showed a 100% sensitivity to predict the need for an intrauterine or neonatal blood transfusion, and a specificity of 67%. Because of the low a priori risk for severe HDFN, the PPV was only 17% using this cut-off; 27% in pregnancies complicated by anti-c, and 9% in the presence of other Rh antibodies.

If repeated titer measurements were performed in alloimmunized pregnancies with a possibly antigen-positive fetus, the risk for exceeding the established cut-off was 18%, varying from 0% for anti-Jk^a/-Jk^b to 36% for anti-c.

A major strength of our study of diagnostic value is that we used a prospectively collected cohort of all pregnancies with maternal RBC alloantibodies detected at a routine first trimester screening. The adherence to the free of charge RBC alloantibody screening program in pregnancy is >99% and the majority of women is screened before Week 13 of pregnancy.(62) For this study, all laboratory data and >90% of clinical outcome data were available. Therefore, the sensitivity of the laboratory tests to predict severe HDFN could be determined very accurately. A limitation might be that this dataset was collected more than 15 years ago. However, we think it still is valid for prediction of treatment with an intra-uterine transfusion, since the treatment guideline did not change. Guidelines to start treatment with exchange transfusion did change, resulting in a higher number of severely ill children treated with intensive phototherapy and fewer with exchange transfusions.(15)

Another limitation of our study might be that in our laboratory—different from other laboratories—for anti-c, -D, and -E, double dose antigen positive RBCs are used for antibody titration. Theoretically, this might result in higher cut-offs for anti-c and -E than for other antibodies, but we did not find such a difference. We also did not find a higher cut-off for anti-c/-E than other studies, using single dose antigen positive RBCs. The cut-off of ≥16 obtained in our study is comparable with the cut-off of 32 from other studies.(5, 59, 159, 161) Titer measurements can vary between laboratories, also with established techniques, in general a comparison can be made with a one-fold dilution difference between technicians.

A limitation of our study concerning the chance of exceeding the cut-offs might be that in 8% of pregnancies with Rh antibodies, other than anti-D, only one sample was sent to the reference laboratory, despite a titer below the cut-off and advised follow-up testing. The reason the advice was not followed is unknown; this may

either because of a miscarriage or preterm birth, or antigen-negativity of the father for the involved antigen, as determined in the primary laboratory. However, this will not substantially change the chance of exceeding the cut-offs.

We made the choice for a cut-off with a 100% sensitivity, but with a PPV of only 17%. If this cut-off is exceeded, the woman should be counseled about her risk for severe HDFN. This risk depends on the antibody specificity, with by far the highest risk in association with anti-c and a small risk in case of other anti-Rh specificities.**6** Although severe HDFN will not occur in the majority of these pregnancies, we think clinical monitoring is justified. This non-invasive monitoring detects the cases needing antenatal and/or postnatal treatment and, on the other hand, reassures the majority of women with a pregnancy without severe HDFN.

The ADCC test had no added value above the titer to perform the initial discrimination between high and low risk for severe HDFN. Cellular assays, such as the ADCC test, are technically demanding and costly, and most laboratories do not perform these assays. However, the contribution of these assays to a more precise risk estimation, can be of added value in pregnancies identified as high risk, such as pregnancies complicated by anti-D or anti-c.(19, 146) It may be that a test analyzing IgG-glycosylation patterns can be a cost efficient surrogate test for the biological activity of RBC alloantibodies.(162)

The aim of antenatal RBC alloantibody testing is to provide obstetric care providers with clinically useful information in the most cost-effective manner.(162) Our previously reported data indicated that RBC alloantibodies with specificities other than anti-D, other Rh specificities or anti-K, rarely induce fetal and neonatal disease. (10) Therefore, in these cases, a single test, producing a low titer, may be sufficient to create sufficient awareness of the care providers that the RBC alloantibody may induce neonatal icterus. For anti-K we recently recommended to use a single anti-K titer measurement and non-invasive fetal K typing for selection of high-risk cases. (160) For anti-D, a titer and a cellular assay, combined with non-invasive fetal D typing, can be used to select high risk cases.

The number of cases with titers above the cut-off was highest for anti-c (36% of cases), but also in case of anti-E (20%) and anti-Fy (12%) these high titers are found. Thus, in about one-third of pregnancies complicated by anti-c, titer measurement predicts a risk for severe HDFN, which will actually occur in about 25 percent of those pregnancies. This makes repeated testing in pregnancy useful. For other types of Rh alloantibodies repeated testing is less useful, since the risk for severe disease is lower, but if the antibody titer is <16 early in pregnancy, we do advise to repeat the laboratory testing, early in the last trimester of pregnancy. This seems sufficient to

differentiate between children at risk for hyperbilirubinemia shortly after birth, and children not at risk.

In case of other RBC alloantibodies, it is important that laboratories involved in antenatal testing, actively inform the care provider to create a sufficient level of awareness to monitor the newborn for the increased risk for hyperbilirubinemia, especially in case of anti-Fy.

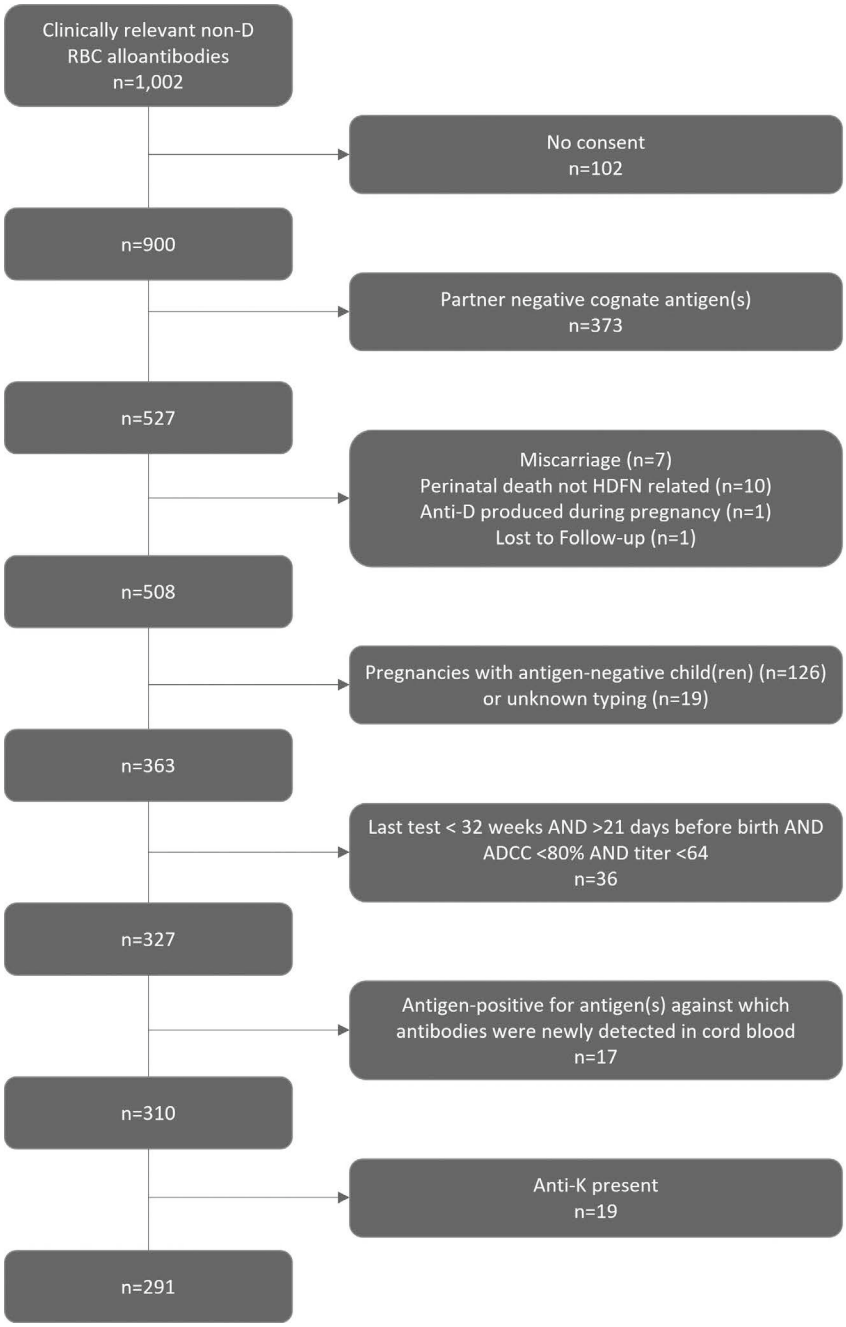
Conclusion

A cut-off of ≥16 for the maximum antibody titer detects all fetuses and children at risk for severe HDFN caused by non-D/non-K RBC alloantibodies. In cases at risk, the ADCC test can be used for a more precise risk estimation. In each pregnancy, one should balance the risk for severe HDFN and the costs, as well as the emotional burden, of repeated laboratory testing.

Repeated testing for anti-c will identify cases with severe HDFN. This is also to be expected for pregnancies complicated by other Rh antibodies. The advice for careful observation because of a risk for neonatal disease should be actively reported by laboratories.

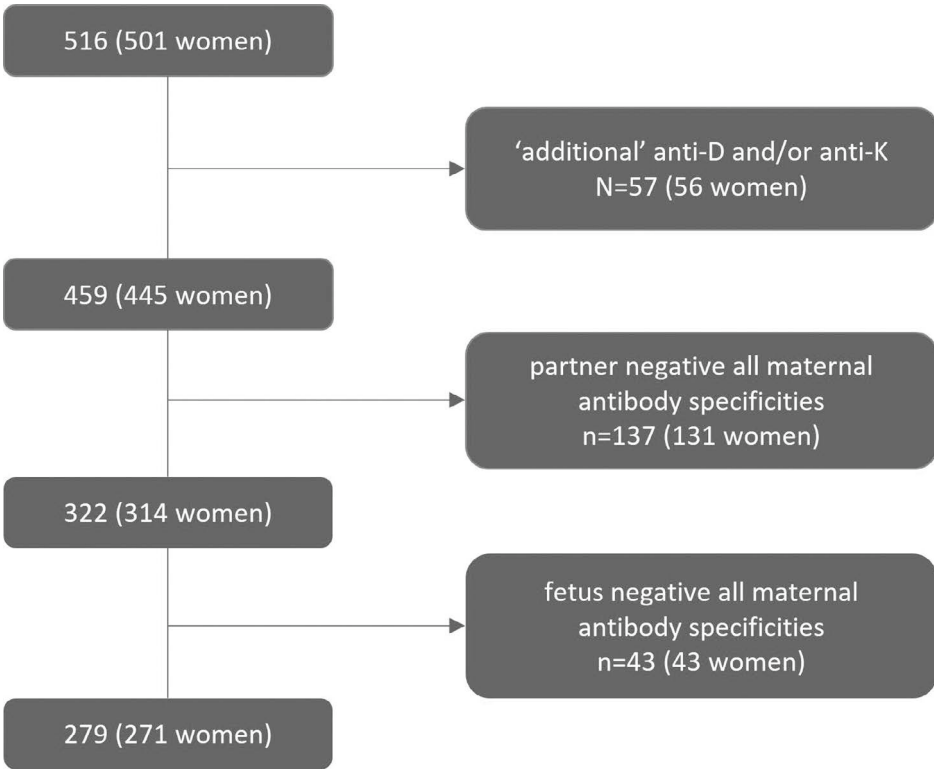
Acknowledgements

We thank all women who participated in the OPZI-study, and we thank obstetric care providers, pediatricians, and collaborators of Sanquin Diagnostic services for collecting clinical and laboratory data.

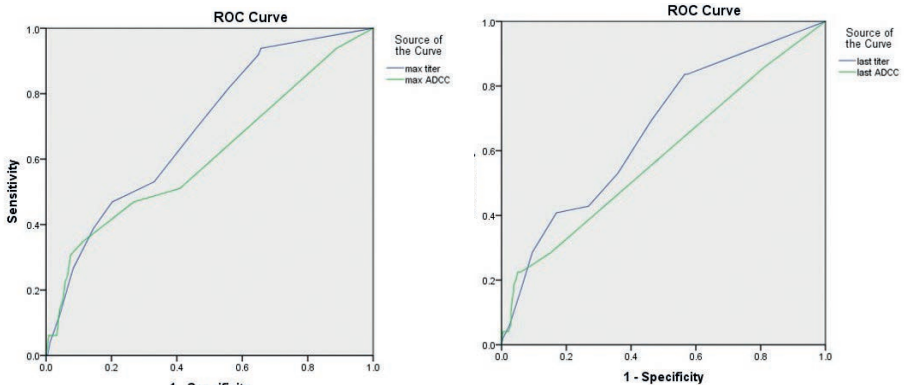


Supplemental Figure 1. *Flowchart of pregnant women with clinically relevant non-D antibodies, detected at first trimester antibody screening in nationwide study in the Netherlands, Sept 1st 2002 until June 1st 2003 & Oct 1st 2003 until July 1st 2004*

Supplemental Figure 2. *Flowchart of pregnancies, at risk for HDFN because of anti-c/-E/-e/-C/Fy^a/-Fy^b/-S/-s/-Jk^a/-Jk^b, detected at Sanquin Diagnostics after positive first trimester antibody screening in 2015-2016*

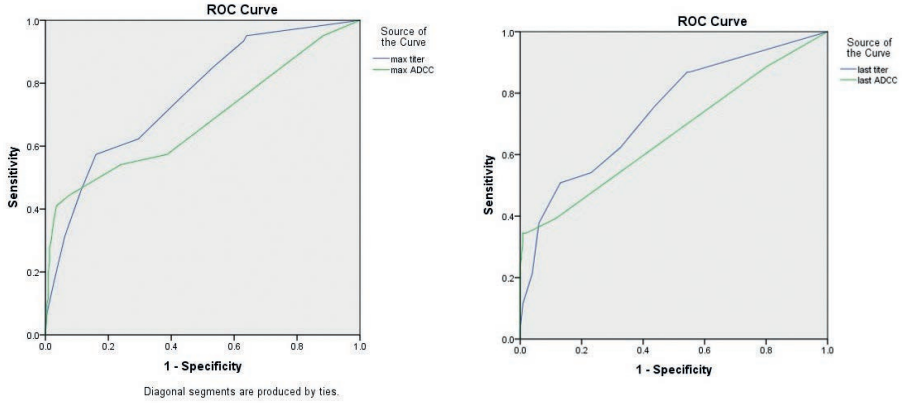


Supplemental Figures 3a,b. *Receiver Operating Curves to predict the need for phototherapy because of HDFN by non-D-/non-K antibodies (n=291) from the maximum and from the last titer and ADCC test result, nationwide study in the Netherlands from Sept 1st, 2002 – June 1st, 2003 & Oct 1st 2003 – July 1st 2004*



Legend: Child positive for the cognate antigen

Supplemental Figures 4a,b. *Receiver Operating Curves to predict the need for antenatal/neonatal transfusion or phototherapy because of HDFN by non-D-/non-K antibodies (n=291) from the maximum and from the last titer and ADCC test result, nationwide study in the Netherlands from Sept 1st, 2002 – June 1st, 2003 & Oct 1st, 2003 – July 1st, 2004*



Legend: Child positive for the cognate antigen

Supplemental table 1. *Prevalence of HDFN in pregnancies at risk for HDFN (child positive for the cognate antigen(s)) because of maternal non-D/non-K RBC alloantibodies, detected at first trimester screening, nationwide study in the Netherlands from Sept 1st, 2002 – June 1st, 2003 & Oct 1st, 2003 – July 1st, 2004*

Specificity RBC antibodies	Antenatal or neonatal transfusion n (%)		Only phototherapy n (%)		No HDFN therapy n (%)		Total	
Anti-c	10 (10.3)	8	24 (24.7)	17	63 (64.9)	50	97	75
. anti-c		2		6		5		13
. anti-c + -E						2		2
. anti-c + -Fy^a				1		4		5
. anti-c + -Jk^a						2		2
. anti-c + -S								
Anti-E	0		13 (16.5)	13	66 (83.5)	66	79	79
. anti-E								
Other Rh	2 (6.3)	1	4 (12.5)	1	26 (81.3)	12	32	13
. anti-C						9		10
. anti-e		1		1		1		2
. anti-C + -e						1		2
. anti-C + Jk^a				2		3		5
. anti-C(w)								
Anti-Fy^a	0		6 (16.7)	6	30 (83.3)	26	36	32
. anti-Fy^a						3		3
. anti-Fy^a + -S						1		1
. anti-Fy^b +-Jk^a								
Other	0		2 (4.3)	1	45 (95.7)	25	47	26
. anti-Jk^a						1		1
. anti-Jk^b				1		10		11
. anti-S						2		2
. anti-s						7		7
Other specificities*								
	12 (4.1)		49 (16.8)		230 (79.0)		291	

**Anti-M (IgG), anti-f, anti-Lu^b, anti-P*

These outcome data were published before in Koelewijn JM, Vrijkotte TG, van der Schoot CE, Bonsel GJ, de Haas M. Effect of screening for red cell antibodies, other than anti-D, to detect hemolytic disease of the fetus and newborn: a population study in the Netherlands. Transfusion 2008;48(5):941-952.

Supplemental table 2. *Number of positive tests, sensitivity, specificity and predictive values to predict the need for antenatal or neonatal transfusion therapy, by cut-off last titer in pregnancies with non-DK RBC antibodies and a child positive for the cognate antigen(s), detected at first trimester screening, nationwide study in the Netherlands, Sept 1st, 2002 – June 1st, 2003 & Oct 1st, 2003 – July 1st, 2004*

n=291		Need for antenatal or neonatal transfusion therapy (n=12)				
Cut-off	Positive tests n	True positive n	Sensitivity % (95% CI)	Specificity % (95% CI)	PPV* % (95% CI)	NPV* % (95% CI)
≥1:8						
all antibodies	86	12	100 (73.5-100)	73.5 (67.9-78.6)	14.0 (11.8-16.5)	100
. anti-c	42	10	100 (69.2-100)	63.2 (52.2-73.3)	23.8 (19.2-29.2)	100
. other Rh	30	2	100 (15.8-100)	74.3 (65.1-82.2)	6.7 (4.9-9.0)	100
≥1:16						
all antibodies	61	11	91.7 (61.5-99.8)	82.08 (77.1-86.4)	18.03 (14.0-23.0)	99.6 (97.2-99.9)
. anti-c	32	9	90.0 (55.5-99.8)	73.6 (63.0-82.5)	28.1 (20.7-37.0)	98.5 (90.9-99.8)
. other Rh	20	2	100 (15.8-100)	83.5 (75.2-89.9)	10.0 (6.8-14.5)	100
≥1:32						
all antibodies	37	9	75.0 (42.8-94.5)	89.96 (85.8-93.2)	24.3 (16.6-34.2)	98.8 (96.9-99.6)
. anti-c	21	8	80.0 (44.4-97.5)	85.1 (75.8-91.8)	38.1 (25.5-52.6)	97.4 (91.4-99.2)
. other Rh	11	1	50.0 (1.3-98.7)	90.8 (83.8-95.5)	9.1 (2.2-31.1)	99.0 (96.1-99.8)

**PPV=Positive Predictive value; NPV=Negative Predictive Value*

Supplemental table 3. *Number of positive tests, sensitivity, specificity and predictive values to predict the need for antenatal or neonatal transfusion therapy, by cut-off maximum ADCC test in pregnancies with non-DK RBC antibodies and a child positive for the cognate antigen(s), detected at first trimester screening, nationwide study in the Netherlands, Sept 1st, 2002 – June 1st, 2003 & Oct 1st, 2003 – July 1st, 2004*

n=291		Need for antenatal or neonatal transfusion therapy (n=12)				
Cut-off	Positive tests n	True positive n	Sensitivity % (95% CI)	Specificity % (95% CI)	PPV* % (95% CI)	NPV* % (95% CI)
≥10%						
All antibodies	124	10	83.3 (51.6-97.9)	59.1 (53.1-65.0)	8.1 (6.2-10.5)	98.8 (95.8-99.7)
. anti-c	57	9	90.0 (55.5-99.8)	44.8 (34.2-55.9)	15.8 (12.4-19.9)	97.5 (85.7-99.6)
. other Rh	36	1	50.0 (1.3-98.7)	67.9 (58.3-76.5)	2.8 (0.7-10.5)	98.7 (94.8-99.7)
≥30%						
All antibodies	28	10	83.3 (51.59-97.91)	93.5 (90.0-96.1)	35.7 (24.95-48.14)	99.2 (97.4-99.8)
. anti-c	20	9	90.0 (55.5-99.8)	87.4 (78.5-93.5)	45.0 (31.2-59.6)	98.7 (92.2-99.8)
. other Rh	2	1	50.0 (1.3-98.7)	99.1 (95.0-100.0)	50.0 (8.4-91.6)	99.1 (96.4-99.8)
≥60%						
All antibodies	11	6	50.0 (21.1-78.9)	98.2 (95.9-99.4)	54.5 (29.9-77.2)	97.9 (96.3-98.8)
. anti-c	9	5	50.0 (18.7-81.3)	97.7 (91.9-99.7)	71.4 (35.7-91.8)	94.4 (90.1-97.0)
. other Rh	2	1	50.0 (1.3-98.7)	99.1 (95.0-100.0)	50.0 (8.4-91.6)	99.1 (96.4-99.8)

** PPV = Positive Predictive Value; NPV = Negative Predictive Value*