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Reassessing critical maternal antibody threshold in RhD alloimmunization: a 16-year retrospective cohort study

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KEYWORDS: alloimmunization; antibody; RhD; transfusion

ABSTRACT

Objective *To determine the critical maternal antibody threshold for specialist referral in cases of RhD alloimmunization.*

Methods *This was a retrospective cohort study, covering a 16-year period at the national tertiary fetal medicine center for management of alloimmunization. Data concerning RhD alloimmunized pregnant women were extracted from an institutional database and maternal anti-D antibody levels were cross-checked with the national reference laboratory. Fetal hemoglobin (Hb) levels were determined only at the first intrauterine transfusion (IUT) and were compared with the pretransfusion maternal anti-D antibody level (IU/mL). Sensitivity, specificity and positive and negative predictive values of maternal antibody thresholds for detecting moderate to severe (Hb ≤ 0.64MoM) fetal anemia were calculated.*

Results *Between 1996 and 2011, 66 women underwent a first IUT for RhD alloimmunization at our institution. The highest serum anti-D antibody level was extracted for 208 RhD alloimmunized women who did not require IUT during the last 10 years of the study period. The traditional maternal antibody threshold of > 15 IU/mL failed to detect 20% of cases of moderate to severe fetal anemia. The ≥ 4 IU/mL threshold had 100% sensitivity but a 45% false-positive rate. The optimal anti-D antibody threshold for specialist referral in our population was ≥ 6 IU/mL; at this level, no case of moderate to severe anemia was missed and specificity was 61%. Use of this threshold would have eliminated 10% of referrals to our fetal medicine unit without compromising fetal outcomes.*

Conclusions *Setting the critical maternal RhD antibody level at >15 IU/mL does not provide sufficient sensitivity. The lower threshold of ≥ 4 IU/mL, though sensitive, is associated with a 45% false-positive rate. In our population, a threshold of ≥ 6 IU/mL minimizes false-positive referrals while maintaining 100% sensitivity for moderate to severe fetal anemia. Copyright © 2014 ISUOG. Published by John Wiley & Sons Ltd.*

INTRODUCTION

While the widespread introduction of Rhesus (Rh) immunoglobulin represents one of the great success stories in modern obstetrics, clinicians continue to encounter cases of RhD alloimmunization[1–3]. In contemporary obstetrics, women with RhD antibodies detected undergo serial antibody level measurements antenatally; those whose levels exceed a certain critical threshold are referred for ultrasound surveillance[1,4]. In the highest-risk cases, in whom middle cerebral artery peak systolic velocity (MCA-PSV) > 1.5 multiples of the median (MoM), who are not candidates for delivery, intrauterine fetal red cell transfusion in a specialist center is recommended[5–7].

Given the increasing rarity of this complication and the use of Doppler ultrasound as the primary surveillance method, routine management of alloimmunization has gradually moved beyond the scope of the general obstetrician into the hands of the fetal medicine specialist. As such, the maternal antibody level that mandates automatic specialist referral becomes critically important. Proper triage based on an appropriate antibody threshold minimizes unnecessary caseload while ensuring that at-risk alloimmunized women are not falsely reassured and denied potentially life-saving fetal therapy.

Twenty years ago, a landmark paper by Nicolaides and Rodeck concluded that serum antibody levels of ≤ 15 IU/mL indicated, at worst, mild fetal anemia which was not likely to require invasive therapy[8]. As such, a threshhold > 15 IU/mL was incorporated into clinical

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practice as warranting fetal specialist opinion. More recently, expert guidelines from the British Committee for Standards in Haematology (BCSH)[9], and endorsed by Scottish National Clinical Guidelines[10], suggest a low, moderate and high risk of hemolytic disease in women with RhD antibody levels of < 4 IU/mL, 4–15 IU/mL and > 15 IU/mL, respectively, and recommend Doppler ultrasound surveillance in moderate- and high-risk cases (i.e. antibody level of ≥ 4 IU/mL).

We undertook a retrospective cohort study of all women with RhD alloimmunization delivering at our institution, the national center for intrauterine transfusion, over the 16-year period between 1996 and 2011. Maternal anti-D antibody levels were compared with fetal hemoglobin (Hb) at the time of first cordocentesis to examine the relationship between antibody levels and fetal anemia in RhD alloimmunization.

SUBJECTS AND METHODS

The National Maternity Hospital, Dublin is a large tertiary obstetric unit, delivering more than 9500 infants annually. We performed the first intrauterine fetal transfusion (IUT) in Ireland in 1990 and are the national referral center for alloimmunization and IUT, serving over four million people. All IUT cases performed in our unit between January 1996 and December 2011 were extracted from our departmental database and cross-checked with the hospital transfusion register. The Irish Blood Transfusion Service, which offers a national referral service for red cell antibody quantification in pregnancy, provided maternal serum antibody levels (IU/mL) for the 10-year period (2002–2011) for those RhD alloimmunized women attending our institution who did not require IUT. This was a retrospective analysis of computerized hospital population data and therefore was deemed exempt from ethical approval from the National Maternity Hospital Research Ethics Committee. The study is reported according to the STROBE statement for cohort studies[11].

All patients in our institution undergo routine blood group antibody screening at the first antenatal visit (i.e. < 14 weeks), with a further serology screening at 28 weeks' gestation for women who are RhD negative. Non-sensitized RhD-negative women receive anti-D immunoglobulin following potentially sensitizing events; routine antenatal prophylaxis at prespecified gestations is not yet standard practice in Ireland. Following detection of maternal RhD antibodies, serial antibody levels are checked every 4 weeks until 28 weeks' gestation and fortnightly thereafter. An anti-D antibody level of ≥ 4 IU/mL warrants referral to our fetal medicine department for subspecialist opinion and surveillance. Since publication of the DIAMOND study in 2006[12] amniocentesis for optical density (ΔOD_{450}) measurements has been completely abandoned in our unit and replaced by non-invasive fetal assessment. Furthermore, although in recent years, RhD-negative women with anti-D antibodies detected have been offered testing for fetal Rh genotype using cell-free fetal DNA techniques[13], this was not routinely performed throughout the 16-year study period.

Data on fetal Hb levels were obtained at the time of cordocentesis. Because the IUT process itself can increase maternal antibody load[13,14], the current study was restricted to fetal Hb levels and maternal antibody levels in women undergoing a first IUT. Data concerning subsequent transfusions or women who had undergone IUT in a previous pregnancy were excluded. In general, a maternal serum sample for anti-D antibody level is collected on the day of IUT, although this was not uniformly the case. Thus, the maternal antibody level used in the current analysis was from the sample taken at the first IUT, or if unavailable, from the most recent antibody level, provided it was within 4 weeks of the first transfusion. Women undergoing transfusion for non-RhD antibodies, including Kell and Rh(c), were excluded. The technique and procedure-related complications for fetal transfusion in our unit have been published previously[15].

Anti-D antibody levels were quantified using the Astoria Pacific International 300 (API 300) continuous flow analyzer (CFA) (Astoria-Pacific, Inc., Clackamas, OR, USA) using R1R1 (CCDDee) group O reagent red cells, a technique first described in 1968[16]. Maternal anti-D binds R1R1 red cells at 37 °C. Sensitized red cells agglutinate and fall out of the suspension and the remaining unsensitized reagent red cells are hemolyzed. Following passage through a flow cell the absorbance measurement (550 nm) is inversely proportional to the level of anti-D (IU/mL). Interassay variability is managed locally by monitoring controls, by testing samples in duplicate and by simultaneous repeat testing of the previous sample. Additional non-RhD antibodies can affect the quantification result and the assay does not distinguish between IgG and IgM. However, these factors can result only in an overestimation of antibody level, but not in underestimation.

For the present study, fetal Hb levels at the time of cordocentesis were adjusted for gestational age by converting to MoM, using data published by Mari *et al.*[17]. Similarly, 'severe anemia', 'moderate anemia', 'mild anemia' and 'no anemia' were defined respectively as Hb level ≤ 0.54 MoM, 0.55–0.64 MoM, 0.65–0.84 MoM and ≥ 0.85 MoM. To allow meaningful statistical analysis, we extracted maternal RhD antibody levels from the 2002–2011 national register determined for 208 RhD alloimmunized women attending our institution who did not require IUT. Women with more than one pregnancy in the national register were included only once, but the highest maternal anti-D antibody level was always taken to accurately represent the highest risk for each patient. Statistical analysis was performed using Statsdirect statistical package 2.7.9 (Statsdirect Ltd., UK).

RESULTS

Between 1 January 1996 and 31 December 2011, 66 women underwent a first transfusion for RhD alloimmunization in our institution. At the time of the first IUT,

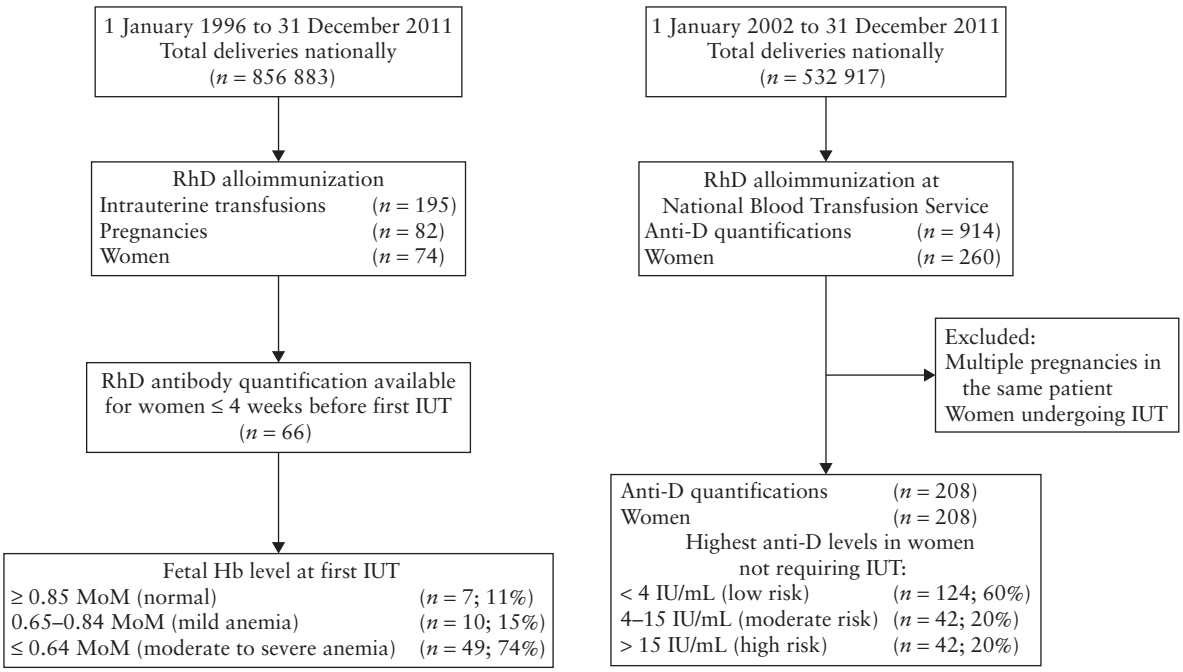

Figure 1 Flowchart showing cohort numbers used in the comparison made in the study. Hb, hemoglobin; IUT, intrauterine fetal transfusion; MoM, multiples of the median; Rh, rhesus.

the degree of fetal anemia was classified as moderate to severe, mild or normal in 74%, 15% and 11% of cases, respectively (Figure 1). Of the fetal Hb levels classified as 'normal' (i.e. ≥ 0.85 MoM), in all but one case fetal Hb was between 0.85 and 0.90 MoM. The last pretransfusion maternal anti-D antibody levels in these 66 cases was compared to the highest levels in 208 women not requiring IUT. In 100% of cases, the pre-IUT antibody level used for analysis was taken ≤ 4 weeks prior to the first IUT; in 73% of cases it was taken ≤ 48 hours prior to IUT. There were no cases of undetected moderate to severe anemia secondary to RhD alloimmunization in non-transfused fetuses in our institution over the 16-year period; i.e. no case of moderate to severe anemia alloimmunization was missed.

Table 1 illustrates the performance of the two most commonly cited critical maternal antibody thresholds, ≥ 4 IU/mL and > 15 IU/mL[8,9]. Although using a referral threshold of ≥ 4 IU/mL resulted in 100% sensitivity in our study (no cases of moderate to severe anemia went undetected), the false-positive rate was 45%. In contrast, setting the referral threshold at > 15 IU/mL, according to Nicolaides and Rodeck[8], raised the specificity for moderate to severe anemia to 75% but resulted in failure to detect 20% (10/49) of moderate to severe cases (Table 1). For those cases of moderate to severe fetal anemia with maternal antibody levels of < 15 IU/mL, the median level was 8.6 IU/mL (range, 6.2–12.3 IU/mL).

In the current study, the optimum maternal RhD antibody level that warranted specialist referral was ≥ 6 IU/mL (6.2 IU/mL). At this threshold, compared to ≥ 4 IU/mL, no cases of moderate to severe fetal anemia were missed in our population (100% sensitivity), while specificity improved to 61% (Table 1). Based on data from the national register, increasing the critical antibody threshold for referral from ≥ 4 IU/mL to ≥ 6 IU/mL would have eliminated 10% of referrals (14/150) for RhD alloimmunization in our population, without missing any instance of moderate to severe fetal anemia (Figure 2). Reassuringly for clinicians managing RhD alloimmunized pregnant women, an antibody threshold of < 4 IU/mL resulted in a 100% negative predictive value (NPV), consistent with expert guidelines; furthermore, in the present study, a maternal

Table 1 Comparison of critical anti-RhD antibody thresholds for the detection of fetal anemia

*Threshold (Study)**	*Degree of anemia*	*Sensitivity (%)*	*Specificity (%)*	*PPV (%)*	*NPV (%)*
≥ 4 IU/mL (Gooch (2007)[9])	Any	100	58	39	100
	Moderate to severe	100	55	33	100
≥ 6 IU/mL (current study)	Any	100	64	43	100
	Moderate to severe	100	61	36	100
> 15 IU/mL (Nicolaides (1992)[8])	Any	81	78	50	94
	Moderate to severe	80	75	41	94

*Only the first author is given. NPV, negative predictive value; PPV, positive predictive value.

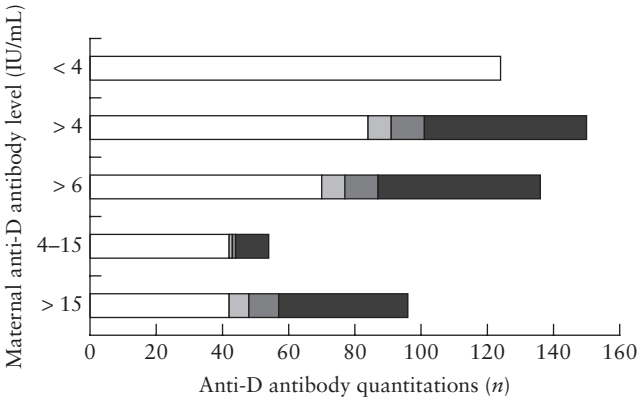

Figure 2 Rates of intrauterine transfusion (IUT) at different maternal anti-D antibody levels in 274 women with RhD alloimmunization. □, No IUT; ▒, IUT without anemia; ▓, IUT with mild anemia; ■, IUT with moderate to severe anemia.

antibody level of < 6 IU/mL was associated with a 100% NPV for moderate to severe anemia (Table 1 and Figure 2).

DISCUSSION

In cases of RhD alloimmunization, there is general agreement that the risk of significant fetal anemia for women with antibody levels of < 4 IU/mL is negligible[8–10,18]. This threshold was originally derived from a study by Bowell *et al.* from Oxford, UK[18]. They reported no instances of cord Hb < 10 g/dL and three cases of exchange transfusion in 78 infants with maternal anti-D antibody levels of < 4 IU/mL, compared to 23/106 infants with a cord Hb < 10 g/dL and 79 with levels > 4 IU/mL who required exchange. Subsequently, in 1992, Nicolaides and Rodeck[8] published a landmark study of 237 alloimmunized pregnant women in whom fetal Hb levels were measured by cordocentesis or fetoscopy and correlated with maternal antibody levels. Amongst 42 women with anti-D antibody levels of < 15 IU/mL, the incidence of moderate to severe fetal anaemia was 0%; thus, the authors concluded that antibody levels of < 15 IU/mL indicated mild anemia only and did not warrant intervention[8]. Although this threshold became established in clinical practice for many years, subsequent expert guidelines suggest referral to a fetal medicine specialist at lower antibody thresholds (≥ 4 IU/mL)[9,10].

Results from the present study appear to corroborate this shift in practice. In our study, which was limited to women undergoing a first IUT for RhD alloimmunization, a referral threshold of > 15 IU/mL would have resulted in missing 20% of cases of moderate to severe fetal anemia, which is clearly unacceptable. Although a threshold of ≥ 4 IU/mL allowed recognition of all moderate to severe cases, we found specialist referral to be unnecessary for women with anti-D antibody levels of 4.0–6.0 IU/mL, representing 10% of referrals. Raising the critical anti-D antibody level to 6 IU/mL would have potential benefits for cost and workload in busy, regional fetal medicine centers and, presumably, would minimize patient anxiety without compromising clinical outcomes.

Non-invasive determination of fetal Rh genotype using fetal DNA extraction from maternal blood is now widely available in many fetal medicine centers, including our own. In many systems, it falls upon the fetal medicine specialist to make fetal Rh genotype testing available for referred women with RhD heterozygous partners, to individualize ongoing management. As such, a critical maternal antibody level for specialist referral is likely to remain important for many years.

It is not clear why 20% of women with moderate to severe fetal anemia in our study had maternal antibody levels of < 15 IU/mL. Many experts agree that maternal antibody levels are a less reliable indicator of fetal anemia after performance of one or more IUTs[1,13,14]. This is because the cordocentesis itself may worsen sensitization (particularly if the needle is passed transplacentally) and also because an increasing fraction of the fetal red cell volume is now donor blood. However, in the present study, we limited our analysis to the most recent maternal anti-D antibody level prior to first transfusion, which should obviate this issue. It has been proposed that women sensitized to alloantibodies in addition to RhD may have higher rates of fetal anemia[19]. However, a recent study from our institution found that the presence of additional non-Kell, non-Rh(c) alloantibodies did not influence outcomes in RhD alloimmunization[15]. Lastly, our group has anecdotally noted a relationship between maternal ABO blood group and risk of fetal anemia in RhD alloimmunization, which is the subject of an ongoing study.

We acknowledge some potential limitations of the present analysis. This was a retrospective study of a large dataset with the possibility of bias inherent in all retrospective studies. However, the fact that the data were collected prospectively in an institutional database and cross-checked with records of our national reference laboratory should minimize the potential for reporting bias. Secondly, given the large numbers involved, it was not possible to extract and analyze every neonatal Hb level in RhD alloimmunized women. While the possibility of an undiagnosed mild neonatal anemia secondary to RhD alloimmunization remains, we are confident that no cases of moderate to severe anemia were missed in our population over the study period. Lastly, while antibody quantifications (IU/mL) are routinely used for RhD alloimmunization in Ireland, many countries, including the USA and Australia, report antibody titers. Although a critical antibody titer is generally considered to be 1:16–1:32 or higher in RhD alloimmunization[1,10] there is no reliable conversion from IU/mL to titer and *vice versa*. This may limit the applicability of our findings in some obstetric populations.

Despite these limitations, this was a large single-institution study at a national referral center for alloimmunization. Antenatal screening and management of women with RhD antibodies were consistent over the 16-year study period. Unlike other studies that tended

to focus on high-risk women following referral to fetal medicine specialists[20,21], our study incorporates antenatal cases of RhD alloimmunization at a national level. Alloimmunized pregnant women with anti-D antibody levels of ≥ 4 IU/mL were managed, and all IUT procedures were performed by a small group of fetal medicine subspecialists throughout the study. While this certainly promotes clinical consistency in patient management, there is always a concern that the results of single-center studies may not be applicable to the wider population. However, the methods we used, both for anti-D antibody quantification and non-invasive ultrasound surveillance, have been widely published[15–17]; we are confident, therefore, that the external validity of our results has not been compromised. While further work is needed to corroborate our findings, we suggest that women with anti-D antibody levels of 4.0–6.0 IU/mL represent a group at lower risk of fetal anemia than those with antibody levels of ≥ 6 IU/mL. Further studies should be undertaken to determine whether women in the 4.0–6.0 IU/mL range indeed require specialist referral or would benefit from a modified program of ultrasound surveillance.

In conclusion, a maternal anti-D antibody threshold of > 15 IU/mL for referral for ultrasound surveillance lacks sufficient sensitivity. The ≥ 4 IU/mL threshold is associated with 100% sensitivity but with a 45% false-positive rate. Our findings suggest that the optimal maternal RhD antibody threshold for predicting moderate to severe fetal anemia is ≥6 IU/mL. Use of this threshold should minimize unnecessary specialist referral without compromising fetal outcomes.

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