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TRANSFUSION

CASE REPORT

A cold case of hemolytic disease of the fetus and newborn resolved by genomic sequencing and population studies to define a new antigen in the Rh system

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Abstract

Background: We report an obstetric case involving an RhD-positive woman who had developed a red blood cell (RBC) antibody that was not detected until after delivery of a newborn, who presented with a positive direct antiglobulin test result. Immunohematology studies suggested that the maternal antibody was directed against a low-prevalence antigen on the paternal and newborn RBCs.

Results: Comprehensive blood group profiling by targeted exome sequencing revealed a novel nonsynonymous single nucleotide variant (SNV) *RHCE* c.486C>G (GenBank MZ326705) on the *RHCE*Ce* allele, for both the father and newborn. A subsequent genomic-based study to profile blood groups in an Indigenous Australian population revealed the same SNV in 2 of 247 individuals. Serology testing showed that the maternal antibody reacted specifically with RBCs from these two individuals.

Discussion: The maternal antibody was directed against a novel antigen in the Rh blood group system arising from an *RHCE* c.486C>G variant on the *RHCE*Ce* allele linked to *RHD*01*. The variant predicts a p.Asn162Lys change on the RhCE protein and has been registered as the 56th antigen in the Rh system, ISBT RH 004063.

Conclusion: This antibody was of clinical significance, resulting in a mild to moderate hemolytic disease of the fetus and newborn (HDFN). In the past, the cause of such HDFN cases may have remained unresolved. Genomic sequencing combined with population studies now assists in resolving such cases. Further population studies have potential to inform the need to design population-specific red cell antibody typing panels for antibody screening in the Australian population.

Brett Wilson and Candice L. Davison contributed equally to this research study and manuscript preparation.

KEYWORDS
blood group antigen, hemolytic disease of the fetus and newborn, RH blood group system, targeted exome sequencing

1 | INTRODUCTION

The Rh blood group system (ISBT 004) is one of the most polymorphic and clinically significant blood groups. A total of 55 antigens were registered in this system as of June 2021, including the extremely immunogenic D antigen and the antithetical C/c and E/e antigens.[1–3] Almost half of these 55 antigens include low-prevalence antigens with many arising from single nucleotide variants (SNV) on the *RHD* or *RHCE* genes, which result in an amino acid change on the RhD or RhCE protein.[4]

Pregnant women who have been alloimmunized to any of the Rh antigens are at risk of being affected by hemolytic disease of the fetus and newborn (HDFN) should the fetus inherit the paternal Rh antigen targeted by the maternal alloantibody. The degree of severity for the HDFN can range from mild, defined by detecting maternal antibody on the cord red blood cells (RBCs), to extremely severe should either fetal or neonatal death occur or in utero transfusions be required.[5] As part of antenatal care strategies to prevent or manage HDFN, blood grouping (ABO and RhD) and red cell antibody screens typically are performed on all pregnant women at the first routine antenatal appointment. The antibody screens are performed using RBC panels, which, though comprehensive, are designed to detect the more frequently encountered antibodies in any given population.

Here we report a case of HDFN involving a woman who had developed a red cell antibody that was not detected until after delivery of the newborn who presented with a mild to moderate HDFN.

2 | CASE PRESENTATION

In 2015, a 35-year-old Australian woman of Indigenous heritage, with no prior history of transfusion, gave birth to a full-term baby after the third pregnancy to her current partner who was also of Indigenous Australian heritage. The mother was blood group A RhD-positive with a negative red cell antibody screen. There had been a history of anti-S, which was no longer detectable. The first pregnancy had resulted in a stillbirth for reasons unknown but possibly related to *E. coli* infection. The second infant was born with severe jaundice requiring phototherapy and exchange transfusion and survived. The reason for the HDFN was unknown but ABO incompatibility was excluded as the infant phenotyped as blood group O. In the current study, a sample taken from the third infant, a male, was referred for immunohematology investigation (see below). Soon after birth, the baby was transferred to the neonatal intensive care unit in case he needed an exchange transfusion and was investigated for septicemia. He received 3 g of intravenous immunoglobulin. Liver function tests showed a total bilirubin of 10 mg/dl with predominance of unconjugated hyperbilirubinemia. The baby was clinically well despite increased bilirubin levels. Infection markers remained all clear.

Standard techniques were used for blood group phenotyping and antibody identification for the immunohematology workup. Reagents were from Immulab (Paragon Care, Parkville, Vic, Australia). The cord cells from the neonate presented with a positive direct antiglobulin test (DAT) using monospecific anti-human globulin (AHG) IgG (4+ reaction) in the manual tube test according to the manufacturer's instructions. The mother and the infant were blood group A, ruling out an ABO incompatibility. The mother's Rh phenotype was D+C+E−c+e+ and infant's D+C+E−c−e+. The maternal plasma and an eluate prepared from the neonate's RBCs, using acid-glycine method, showed negative reactions with RBC typing panels used in routine antibody detection tests. In contrast, the maternal plasma reacted strongly with the paternal RBCs (Group O, D+C+E+c+e+) by a tube polyethylene glycol (PEG) indirect antiglobulin test (IAT) method. The maternal plasma also reacted with the neonate's RBCs after bound IgG antibody had been removed from the DAT positive cells, using the acid-glycine eluate method. The eluate from the neonate's RBCs also reacted with the paternal RBCs. These results together suggested the presence of an antibody in the maternal plasma reacting to a low-prevalence antigen on the paternal and infant RBCs.

The maternal plasma sample was crossmatched with an extended RBC panel of low-prevalence antigens, Table 1. All results were compatible, excluding these antigens as possible candidates for the specificity of the maternal antibody.

The paternal RBCs were phenotyped using monoclonal/polyclonal antibodies specific for additional low-prevalence antigens, Table 1, to identify a possible antigen specificity for the maternal antibody. However, the paternal red cells failed to react with any of the antibodies

TABLE 1 Serological typing for low-prevalence antigens[a]

Maternal plasma failed to react with RBC panel expressing the following blood group antigens	002 MNS	Mur+, M^g+, Mt(a+), Ri(a+), Mit+,
	004 RH	V+, E^w+, VS+, JAHK+
	005 LU	Lu13+,
	010 DI	Rb(a+), Wu+, Tr(a+), Fr(a+),
	013 SC	Sc2+, Rd(a+),
	016 LW	LW(a+b+),
	020 GE	Ls(a+), Dh(a+),
Father's RBCs failed to react with antibodies to the following blood group antigens	002 MNS	Mi^a, Mur, Vr, Mt^a, Ri^a, Ny^a, Hut, Dantu, MNTD, Vw
	004 RH	C^w, C^x
	005 LU	Lu^a
	006 KEL	Kp^a, K17
	010 DI	Di^a, Wr^a, Wd^a, ELO, Wu, Bp^a, Mo^a, Hg^a, Vg^a, Sw^a, BOW, Tr^a
	011 YT	Yt^a
	013 SC	Rd
	015 CO	Co[b]
	020 GE	Wb
	Other[b]	Bx^a, By, Pt^a

[a]Antigens are grouped within respective blood group systems using ISBT blood group system numerical and system symbol names, for example, ISBT 002 MNS.

[b]These antigens are low-incidence antigens in the ISBT 700 series and not yet assigned to any blood group system as the genetic basis is unknown.[4]

represented. Paternal RBCs treated with trypsin, chymotrypsin, chloroquine, papain, and 0.2M DTT had no effect on the paternal RBC antigen expression, which still tested positive against the maternal plasma.

Genotyping was performed on maternal, paternal, and infant genomic DNA using the SNP-based Immucor PreciseType™ Molecular BeadChip™ HEA, RHD, and RHCE tests but did not signal any variants of note. Massively parallel sequencing (MPS) and data analysis were performed using the TruSight One Sequencing Panel (TSO; Illumina Inc., San Diego, CA) as previously described.[6,7] Manual analysis of the variant call file revealed both the father, defined here as the proband, and infant, but not the mother, were heterozygous for nucleotide substitution (c.486C>G) on the *RHCE* gene, Figure 1A,B. This predicts a missense amino acid change p.Asn162Lys on the RhCE protein.

The Illumina TSO sequence reads are only short fragments and here did not define which *RHCE* allele the variant is on. However, we can deduce from the infant phenotype profile (D+C+E−c−e+) that the infant is homozygous for *RHCE*Ce* (Ce/Ce) and therefore the c.486C>G variant is on *RHCE*Ce* allele Figure 1B. We can further deduce that *RHCE*Ce* and *RHD*01* are traveling together as *RHD* copy number variation (CNV) analysis of the MPS sequencing data showed that both the father and infant are homozygous for the *RHD*01* allele. However, it was not possible to conduct an extended family study to test whether the maternal antibody reactivity pattern correlated with segregation of the *RHCE*Ce* c.486C>G variant allele in other family members.

3 | BLOOD GROUP GENOMIC POPULATION STUDY

In 2018, a population study commenced to profile blood groups in Indigenous Australians. Blood samples were kindly provided by 247 Indigenous Australians who are community members of the Carbal Medical Services in Toowoomba, Queensland. This study was conducted with informed consent under Australian Red Cross Lifeblood (Lifeblood) HREC ethics approval, 2018#17. ABO and Rh phenotyping were performed using accredited laboratory procedures and the remaining RBCs were kept frozen. Targeted blood group exome sequencing was performed using a custom-designed panel as previously described.[8] Briefly this panel targeted the 5′-untranslated regions and exons for 43 genes associated with 36 RBC blood group systems from ISBT 001 to ISBT 036, plus the additional erythroid transcription factor genes *KLF1* and *GATA1*. DNA libraries were prepared using the Nextera rapid-capture custom enrichment protocol. Samples ($n = 12$) were indexed, pooled, and sequenced on an Illumina MiSeq (Illumina Inc.) using standard V2 chemistry and flow cells. Binary alignment map (BAM) and variant call format (VCF) files were generated onboard the MiSeq using MiSeq Reporter v2.5.1.3 mapping against the human reference genome GRCh37/hg19. Analysis of VCF files and CNV analysis was performed manually. Sequencing showed two individuals (2/247) were heterozygous for the SNV, *RHCE* c.486C>G, observed in the proband and infant from the 2015 HDFN case. No other variants of note were identified on the *RHCE* allele.

One sample was hemizygous for *RHD* by CNV analysis with an observed serology phenotype D+C+E−c+e+. The other was homozygous for *RHD*, phenotype D+C+E+c+e+. All antigens reacted strongly with a score of 12 (0–12 agglutination score) with Rh monoclonal typing reagents except for one weakened reactivity score of 10 documented for the e type.

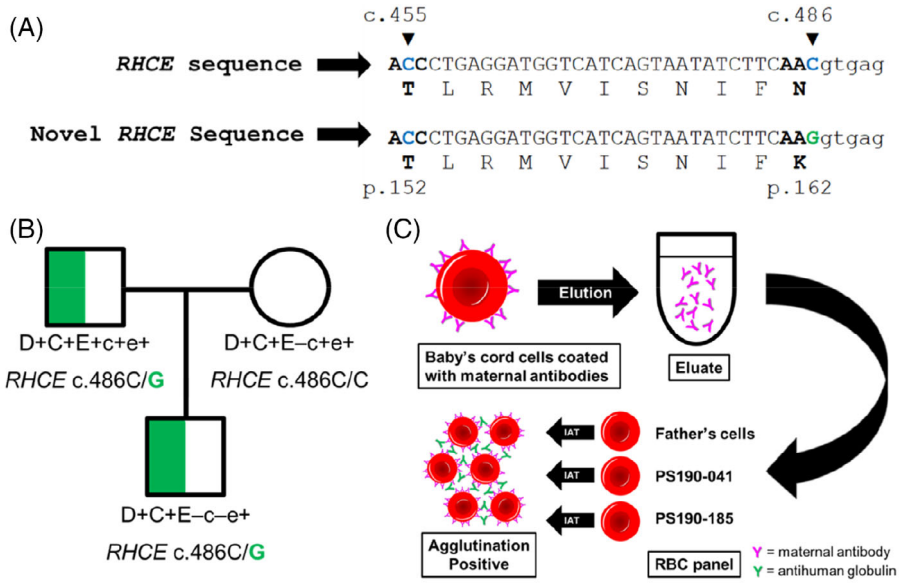

FIGURE 1 Serological and molecular investigations showing a novel c.486G variant on the *RHCE* allele, with paternal inheritance and with maternal antibody reactivity associating with red cells from carriers of this variant. *RHD* zygosity obtained from copy number variation (CNV) analysis of MPS sequencing data. Figure 1A. Schematic representation of *RHCE* sequences comparing the reference sequence, (NG_009208.3 on the top line) with sequence showing novel *RHCE* c.486C>G (p.Asn162Lys or p.N162K) variant. Nucleotides in capital and small letters indicate exonic and intronic regions, respectively. The predicted amino acid translation and position are shown directly under the nucleotide sequence. Inverted triangles indicate nucleotide positions with c.486 indicating the novel SNV. Diagram shows c.486C>G *in cis* with c.455C (p.Thr152 or p.T152). Cytosine at c.455 is specific for *RHCE* gene—in *RHD* gene, it is c.455A (p.Asn152 or p.N152) thus showing the novel SNV is on the *RHCE* allele. A screenshot of the sequence alignment is shown in Figure S1. Figure 1B. Pedigree chart for the trio (father, mother, and baby). Chart shows the inheritance of the *RHCE* c.486C>G variant, indicated in green text and green shade, from the father to baby. Figure 1C. Antibody eluted from the baby's cord cells agglutinated RBCs from the father and two study participants all carrying the *RHCE* c.486C>G variant.

Frozen RBCs from these two Indigenous Australians were recovered for further serology testing with the maternal plasma and cord eluate from the 2015 case (both also having been stored frozen). The RBCs from both individuals, with the *RHCE* c.486C>G variant, were ABO compatible with the maternal sample, allowing a direct crossmatch to be performed. The crossmatch showed that the maternal plasma was incompatible with RBCs from both Indigenous Australian study samples with the *RHCE* c.486C>G variant. Standard antibody and RBC adsorption and elution techniques were used to further test the specificity of the maternal antibody. Maternal plasma was adsorbed and eluted from RBCs for one of the Indigenous Australian study samples. The eluate, as well as original eluate from the neonate's RBCs, reacted with the paternal RBCs as well as with RBCs from the two study individuals, Figure 1C.

The *RHCE*Ce* c.486C>G variant was absent from public databases, ExAC, dbSNP, and GnomAD and also from 656 Australian blood donor samples tested on the targeted blood group exome sequencing system.[8] This *RHCE*Ce* c.486C>G variant was assigned GenBank MZ326705.

4 | DISCUSSION AND CONCLUSION

This case report defines a new antigen in the Rh system where the associated antibody in the alloimmunized mother resulted in a mild to moderate HDFN. The complementary application of genomic and serological techniques helped define this antigen in a three-step process. First, comprehensive blood group profiling by targeted exome MPS implicated a nonsynonymous SNV in the *RHCE*Ce* (*RHCE*02*) allele in the father and baby. Second, an Indigenous Australian population study revealed the same SNV in two further individuals. In the third step, stored RBCs permitted follow-up serology testing to show that the maternal antibody (from plasma and cord eluate) reacted specifically with RBCs from the two individuals from the Indigenous Australian population study.

The *RHCE* c.486C > G variant is novel as it has not been listed on any public database previously. This variant occurs at the splice site junction between exon 3/intron 3 (Figure S1). However, this did not appear to impact on splicing as no weakening of antigen expression was observed by serology, except for one reduced score

from 12 to 10 for one sample. The variant predicts a p.Asn162Lys change on the third of the six extracellular loops for the RhCE protein. A model using the SIFT tool predicts that the impact of this change on the Rh protein will be "Tolerated". However, this modeling does not necessarily predict the immunogenic potential for the amino acid change. This blood group antigen was acknowledged by the ISBT Working Party for Red Cell Immunogenetics and Blood Group Terminology in June 2021 as fulfilling the criteria for assigning a new blood group antigen and is now registered and named CETW.[9] (CETW is named after the *RHCE* allele and the town, Toowoomba, where the collaborating Carbal Medical Centre is based). CETW becomes the 56th antigen in the Rh system and is numbered ISBT 004063, noting seven antigens were previously made obsolete.

While the *RHCE* c.486C>G variant reported here is novel, a synonymous *RHCE* variant at the same site, c.486C>T, is reported (rs199725473) with a frequency MAF of 0.000040 (10/251492, GnomAD) of 0.000033 (4/121408, ExAC). Therefore, this position on the gene appears to be a triallelic site. It is interesting that the c.486G variant has not been detected before on any public database. Given that it has now been described in three cases here it suggests that the variant may be specific among the Indigenous Australian population.

A recent review summarized the low-prevalence RH antigens that have been associated with severe or extremely severe HDFN.[5] Many involve single amino acid changes, which are predicted to reside on the extracellular domain of the protein.[5,10,11] Among the earliest reported antigen was an antigen called Berrens or Be^a, which was discovered through an HDFN case in 1953 and is capable of causing severe HDFN.[12] While a large family study reportedly suggested this antigen belonged to the Rh system the genetic basis was not defined until 2010. In 2010, cDNA sequencing studies on samples from four separate probands[13] revealed an *RHCE*ce* allele (linked to *RHD*01N*), with a c.662C>G change predicting a p.Pro221Arg change on the fourth extracellular loop of the RhCE protein. The antigen was defined as RH 004036. The limit of our study is that neither cDNA nor cell expression studies were possible. However, the phenotype and genotype distribution pattern in our study shows the variant is associated with the *RHCE*Ce* allele linked to *RHD*01*.

The Berrens case noted above illustrates the extent of delays, sometimes involving decades, between reporting an antigen and defining the genetic basis that occurred in the past. It is of note therefore that genomic-based technologies and tools are now available to resolve the cause of the cases presenting with unexplained antibodies to RBC antigens and this has been exemplified recently in the Australian context.[14,15] Resolution of these cases has clinical relevance, providing counseling for the family affected and, through precision medicine, providing approaches to manage future pregnancies for the alloimmunized woman. For example, with nano-based technologies such as droplet-digital PCR and genomic sequencing technologies, it will be feasible to apply non-invasive prenatal tests (NIPT) to predict whether the fetus has inherited the paternal RBC antigen and is at risk of HDFN.[16,17] This knowledge in turn guides whether intensive antenatal ultrasound monitoring will be required to check for and treat early warning signs of fetal anemia.

In conclusion, this study defines a new antigen in the Rh blood group system observed in a small cohort of Indigenous Australians. Given that maternal alloantibodies against low-prevalence RBC antigens can go undetected during antenatal screening further studies may well recommend the need for including population-specific RBC screening panels for antenatal management in the Australian population.

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CONFLICT OF INTEREST

The authors have disclosed no conflicts of interest.

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REFERENCES

1. Peyrard T, Wagner F. The Rh system. In: Cohn CS, Delaney M, Johnson S, Katz L, editors. Technical manual. Bethesda Maryland United States: AABB; 2020. p. 329–54.
2. Avent ND, Reid ME. The Rh blood group system: a review. Blood. 2000;95:375–87.
3. Wagner FF, Flegel WA. Review: the molecular basis of the Rh blood group phenotypes. Immunohematology. 2004;20:23–36.
4. ISBT Terminology Committee. Red cell immunogenetics and blood group terminology [monograph on the internet]. Amsterdam: International Society of Blood Transfusion; 2022. Available from: http://www.isbtweb.org/working-parties/red-cell-immunogenetics-and-blood-group-terminology/
5. Floch A. Maternal red blood cell alloimmunisation working party, literature review. RH blood group system: rare specificities. Transfus Clin Biol. 2021;28:314–20.
6. Schoeman EM, Lopez GH, McGowan EC, Millard GM, O'Brien H, Roulis EV, et al. Evaluation of targeted exome sequencing for 28 protein-based blood group systems, including the homologous gene systems, for blood group genotyping. Transfusion. 2017;57:1078–88.
7. Schoeman EM, Roulis EV, Liew YW, Martin JR, Powley T, Wilson B, et al. Targeted exome sequencing defines novel and rare variants in complex blood group serology cases for a red blood cell reference laboratory setting. Transfusion. 2018;58:284–93.
8. Roulis E, Schoeman E, Hobbs M, Jones G, Burton M, Pahn G, et al. Targeted exome sequencing designed for blood group, platelet, and neutrophil antigen investigations: proof-of-principle study for a customized single-test system. Transfusion. 2020;60:2108–20.
9. Gassner C, Castilho L, Chen Q, Clausen FB, Denomme GA, Flegel WA, et al. International Society of Blood Transfusion Working Party on Red Cell Immunogenetics and Blood Group Terminology Report of Basel and three virtual business meetings: Update on blood group systems. Vox Sang. 2022;117:1332–1344.
10. Floch A, Pirenne F, Barrault A, Chami B, Toly-Ndour C, Tournamille C, et al. Insights into anti-D formation in carriers of RhD variants through studies of 3D intraprotein interactions. Transfusion. 2021;61:1286–301.
11. Omasits U, Ahrens CH, Müller S, Wollscheid B. Protter: interactive protein feature visualization and integration with experimental proteomic data. Bioinformatics. 2014;30:884–6.
12. Davidsohn I, Stern K, Strauser ER, Spurrier W. Be, a new private blood factor. Blood. 1953;8:747–54.
13. Hue-Roye K, O'Shea K, Gillett R, Wadsworth LD, Hume H, Barnes J, et al. The low prevalence Rh antigen Be(a) (Rh36) is associated with RHCE*ce 662C>G in exon 5, which is predicted to encode Rhce 221Arg. Vox Sang. 2010;98:e263–8.
14. McBean RS, Hyland CA, Hendry JL, Shabani-Rad MT, Flower RL. SARA: a "new" low-frequency MNS antigen (MNS47) provides further evidence of the extreme diversity of the MNS blood group system. Transfusion. 2015;55:1451–6.
15. Lopez GH, Wilson B, Millard GM, Cawthorne TL, Grey DE, Fong EA, et al. A new high-prevalence LW antigen detected by an antibody in an Indigenous Australian homozygous for LW*A c.309C>A variant. Vox Sang. 2022;117:958–965.
16. Tsui NB, Hyland CA, Gardener GJ, Danon D, Fisk NM, Millard G, et al. Noninvasive fetal RHD genotyping by microfluidics digital PCR using maternal plasma from two alloimmunized women with the variant RHD(IVS3+1G>A) allele. Prenat Diagn. 2013;33:1214–6.
17. O'Brien H, Hyland C, Schoeman E, Flower R, Daly J, Gardener G. Non-invasive prenatal testing (NIPT) for fetal Kell, Duffy and Rh blood group antigen prediction in alloimmunised pregnant women: power of droplet digital PCR. Br J Haematol. 2020;189:e90–e4.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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