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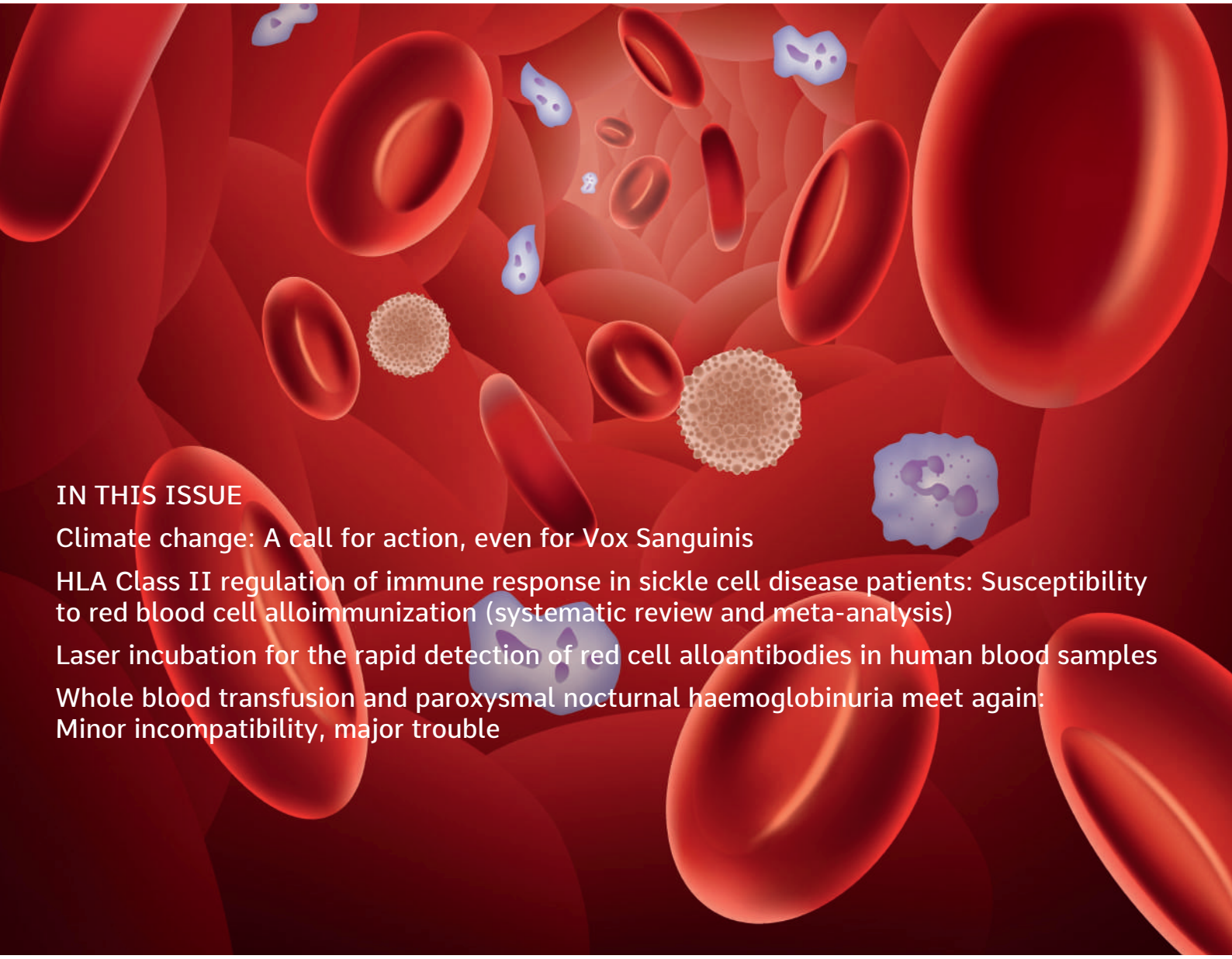

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International Journal of Blood Transfusion

Official Journal of the International Society of Blood Transfusion

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Vox Sanguinis

International Journal of Blood Transfusion

Aims and Scope

Vox Sanguinis reports on all issues related to transfusion medicine, from donor vein to recipient vein, including cellular therapies. Comments, reviews, original articles, short reports and international fora are published, grouped into 10 main sections:

1. Blood Component Collection and Production: Blood collection methods and devices (including apheresis); blood component preparation; inventory management; collection and storage of tissues; quality management and good manufacturing practice; automation and information technology; plasma fractionation techniques and plasma derivatives;
2. Cellular Therapy: Cell-based therapies; CAR T-cell therapies; genetically modified cell therapies; cellular therapy (sources; products; processing and storage); stem cells; cell-based regenerative medicine; cellular immunotherapy; molecular therapy;
3. Donors and Donations: Donor recruitment and retention; donor selection; donor health
4. Haemovigliance: Adverse events in blood and blood component donors and transfusion recipients; corrective and preventive measures of complications; near-misses and errors in the transfusion chain; evaluation and outcomes of adverse events
5. Immunohaematology and Immunogenetics: autoimmunity in haematology; alloimmunity of blood; pre-transfusion testing; complement in immunohaematology; blood phenotyping and genotyping; genetic markers of blood cells and serum proteins: polymorphisms and function; parentage testing and forensic immunohaematology;
6. International Forum: Section in which topics related to any aspects of the transfusion chain (from technical to scientific, including socioeconomic and ethical facets) are discussed by invited participants and summarized by guest editors
7. Patient Blood Management: Bloodless surgery; preoperative anaemia; minimizing blood loss during surgery; alternatives to blood transfusion;
8. Review: Comprehensive reviews or short comments of any aspect related to transfusion medicine;
9. Transfusion Medicine: Transfusion indication, transfusion practice, thresholds and audits; transfusion efficacy assessment, clinical trials; therapeutic apheresis;
10. Transfusion-transmitted Disease and its Prevention: Identification and epidemiology of infectious pathogens transmissible by blood; donor testing for transfusion-transmissible infectious pathogens; bacterial contamination of blood components; pathogen inactivation;

This comprehensive coverage has made the journal essential reading for a wide range of specialists interested in the present state of transfusion research and practice.

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EDITORIAL

Vox Sanguinis ISBT International Society of Blood Transfusion

Climate change: A call for action, even for *Vox Sanguinis*

Between the 6th and 18th November 2022, the government of the Arab Republic of Egypt will host the 27th Conference of the Parties (COP) of the United Nations (UN) Framework Convention on Climate Change in the city of Sharm El-Sheikh. This conference will aim to continue to make progress on controlling climate change and its negative impact around the globe [1]. According to the UN, climate change is a global emergency that extends beyond national borders and requires international cooperation and coordinated action at all levels.

An expression of the international cooperation in this field is the Paris Agreement that entered into force on 4th November 2016 after approval in 2015, and that, so far, has been joined by 192 countries. It includes, among other things, a commitment from all countries to reduce their greenhouse gas emissions. The main aim is to limit the global temperature increase this century to 2°C while pursuing efforts to limit the increase even further to 1.5°C. The agreement creates a pathway for developed nations to support developing countries in their climate mitigation and adaptation efforts [2].

The reader of *Vox Sanguinis* might wonder why we are dealing with this topic in a scientific journal whose main scope is blood transfusion. This editorial is justified for two reasons. The first is that over 200 journals around the world have just published an editorial, asking for urgent action for Africa and the world [3]. Ahead of the meeting, the editorial wishes to 'call for urgent action to ensure it is the COP that finally delivers climate justice for Africa and vulnerable countries. This is essential not just for the health of those countries for but for the health of the whole world'.

The call is clearly timely. Climate change is taking its toll of victims not only in Africa due to the severe droughts that in recent years have affected sub-Saharan Africa but also in other parts of the world. In 2022, monsoon rains, combined with melting glaciers that followed a severe heat wave, have caused the worst flooding in Pakistan in the country's history, affecting 33 million people and killing 1569. Pakistan is yet another example of the sad contrast between very high human development index (HDI) countries and countries with low and very low HDI; Pakistan contributes less than 1% of global greenhouse gas emissions, but is one of the places most vulnerable to climate change [4].

The second reason for this editorial is that climate change is causing significant variations in the environment, including in a field that has presented a challenge for transfusion medicine specialists since the early days of our practice, namely, the fight against infectious diseases that are potentially transmitted by transfusion. These environmental variations have been caused by (1) the changes in the ecology of the vectors able to transmit infections to humans and (2) the direct consequences of climate change, including the increase in temperature and flooding.

Regarding the changes in the ecology of the vectors, a study published this year estimated that at least 10,000 virus species can infect humans but, at present, the vast majority are circulating silently in wild mammals; however, changes in climate and land use can lead to zoonotic spillover. The authors predicted that, by the year 2070, there would be a 4000-fold increase in cross-species transmission of their associated viruses. Because of their unique dispersal ability, bats account for most of the novel viral sharing and are likely to share viruses along evolutionary pathways that will facilitate future emergence in humans [5]. As a matter of fact, for some authors, SARS-CoV-2 was an example of such possible spillovers [6].

The second effect of climate change on the transmissibility of infectious diseases is the consequences of climate hazards, such as the rise in temperature, which will increase mosquito survival and biting rates, or the floods that will cause an upsurge in the transmission of waterborne infections [6]. In fact, Mora et al. reported that climate change has aggravated 218 (58%) of the 375 infectious diseases listed in the Global Infectious Diseases and Epidemiology Network (GIDEON) and the US Centres for Disease Control and Prevention National Notifiable Diseases Surveillance System [7].

It is clear that climate change will transform not only the planet but also our practice in the field of transfusion medicine both because an increase in the number of old/new infectious diseases potentially transmitted by transfusion might occur and we will have to develop strategies to prevent their transmission, and also because, even with no evidence of transmissibility by transfusion, a new infectious disease has the potential to cause huge disruption to the functioning of blood establishments and hospital transfusion departments; SARS-CoV2 is a case in point.

Given this situation, it seems reasonable that we, all of us, try to minimize the increase in greenhouse gases, mainly CO_2, generated by our own activities. In our case, the publishing of *Vox Sanguinis* has an impact on our environment. For this reason, from January 2023 onwards, the printing and distribution of *Vox Sanguinis* will be moved from Singapore to the United Kingdom. With this transfer, we estimate the distance travelled by the printed journals to the desks of International Society of Blood Transfusion members will decrease from 19,310,295 to 10,865,606 km, which will represent a reduction in the CO_2 footprint from 32.6 to 19.1 tons, a 41.4% reduction (Claire Dowbekin, personal communication). In addition, we are considering other measures to further decrease the CO_2 footprint associated with the publication and reading of *Vox Sanguinis*.

As UN Secretary-General António Guterres expressed it in the opening of the 2022 General Debate, 'We must end our suicidal war against nature', and perhaps we might contribute to the beginning of the end of the war by decreasing the CO_2 footprint of the publication and distribution of our journal, *Vox Sanguinis*.

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Miquel Lozano

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Vox Sanguinis

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REVIEW

Vox Sanguinis ISBT International Society of Blood Transfusion

HLA Class II regulation of immune response in sickle cell disease patients: Susceptibility to red blood cell alloimmunization (systematic review and meta-analysis)

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Abstract

Background and Objectives: Sickle cell disease (SCD) patients are commonly treated with red blood cell (RBC) transfusion. Pretransfusion tests commonly involve limited serological antibody testing. RBC alloimmunization to RBC antigens is a frequently encountered complication seen in chronically transfused patients. Genetic factors such as the human leukocyte antigen (HLA) are known to influence and regulate immune responses. HLAs are highly polymorphic and play an essential role in regulating immune responses, including RBC alloimmunization. The aim of this study was to conduct a systematic review and meta-analysis to evaluate the association between HLA Class II allelic polymorphisms with the possible risk of developing RBC alloantibodies.

Materials and Methods: Four databases were systematically searched for relevant studies between the years 2000 and 2021 following the PRISMA guidelines. Four articles met the eligibility and quality criterion, and three alleles, HLA-DRB1*04, HLA-DRB1*15 and HLA-DQB1*03, that were found to be potentially associated with an increased risk in alloantibody formation were included.

Results: The primary outcome measure was alloimmunization by RBC antigen exposure in multiply transfused SCD patients. The total estimate of alloimmunization of the SCD patients was 2.33 (95% CI, 1.58–3.44), demonstrating susceptibility to RBC alloantibody formation. Heterogeneity between the studies was insignificant, suggesting the differences associated with random sampling errors. The results showed that SCD patients carry an increased risk of producing RBC alloantibodies.

Conclusion: A strategy to prevent RBC alloimmunization is genotyping for genetically susceptible SCD patients receiving multiple transfusions. Early identification of genetic variants that can potentially increase the risk of RBC alloimmunization could aid in the screening process and selection of phenotypically matched RBC units.

Keywords

HLA Class II, RBC alloimmunization, sickle cell disease

Highlights

- HLA Class II alleles are associated with susceptibility to alloimmunization in multiply transfused patients with sickle cell disease (SCD).
- The variants HLA-DRB1*04, -DRB1*15 and -DQB1*03 confer an increased risk of producing alloantibodies.
- Early identification of genetic factors affiliated with likely alloantibody development would allow selective use of phenotypically matched red blood cell units in genetically susceptible SCD patients.

INTRODUCTION

Background

Sickle cell disease (SCD) is a hereditary condition affecting haemoglobin and blood flow within the body leading to pain and organ failure [1]. SCD is part of a wider group known as haemoglobinopathies and is most common in people of African ancestry. Transfusion of blood products is utilized as a supportive treatment of haemoglobinopathies to reduce morbidity and mortality [2]. Transfusion therapy can be highly beneficial to patients if the necessary safety precautions are considered. Unfortunately, the consequences of transfusions may still occur despite these precautions. SCD patients are highly susceptible to red blood cell (RBC) alloimmunization as they become reliant on blood transfusions (receiving multiple transfusions within their lifetime) to maintain a relatively healthy life.

Among all transfused recipients, alloimmunization to RBC antigens usually occurs in about 2%–5% [3]. However, approximately 40%–50% of the transfused SCD patients develop RBC alloantibodies in the absence of RBC antigen phenotype matching beyond ABO and RhD matching [4, 5]. Alloimmunization to RBC antigens in SCD patients has a significantly higher rate than other chronically transfused patient populations, potentially due to the differences in RBC antigen expression frequencies between people of different ethnic backgrounds (antigen disparity), high transfusion burden, genetic diversity and immune system considerations etc. [3, 4]. As a result, RBC alloimmunization can complicate the selection of future compatible transfusion units and furthermore, increase the risk of haemolytic transfusion reactions (HTRs), and the development of additional RBC alloantibodies and autoantibodies in subsequent transfusions [6–8].

Significance of RBC alloantibodies in polytransfused SCD patients

The formation of alloantibodies and autoantibodies to RBC antigens has the potential to cause clinically significant complications that promote haemolysis, iron overload and RBC alloimmunization [2, 9]. In SCD patients, the most common alloantibodies formed by multiple transfusions are anti-C and anti-E in the Rh blood group system and anti-K from the Kell blood group system [2]. Therefore, SCD patients are usually transfused with ABO and RhD-compatible, and phenotype matched with anti-C, -E and -K negative RBCs as a preventative measure. Alloimmunization is less prevalent in populations where there is higher phenotypic compatibility between the donor and SCD recipient [10–12]. This is supported by the study conducted by Natukunda et al demonstrating a lower rate of RBC alloimmunisation in the Ugandan SCD population [12].

Role of HLA in alloimmunization

Some studies have demonstrated that alloimmunized SCD patients show a worse survival rate than non-alloimmunized patients [13, 14]. It is not known why some patients become alloimmunized while others do not. This has raised questions about the contributions to the high rate of alloimmunization in SCD populations. However, it is known that the immune response is regulated by genetic factors such as human leukocyte antigen (HLA), having the ability to influence alloimmunization [5, 14, 15]. HLA genes are highly polymorphic and are involved in T-cell-mediated immunity [16]. HLA Class II molecules process and present RBC antigens by antigen-presenting cells (APCs) to T-cell receptors (TCR), which, in turn, activate CD4 helper T-cells. This involves the interaction between T and B cells and the differentiation of B cells into plasma cells. CD4+ regulatory T-cells (Tregs) are immune response modulators; the activation of T-cells triggers Tregs to suppress the activation and proliferation of multiple cell types. A study has found that the Treg suppressive function is reduced in antibody responders in SCD patients [17]. Therefore, resulting in alloimmunization to RBC antigens. It is hypothesized that inheriting certain HLA alleles can predispose patients to RBC alloimmunization (Figure 1) [10].

A relationship between HLA polymorphisms and immune responses to various RBC antigens has been established, and these include Duffy^a [18, 19], Kell [20], Diego [21] and Kidd^a [22]. Noizait-Pirenne et al conducted a study to identify the HLA-DRB1 restriction molecules within the anti-Fy^a and anti-K groups in Caucasians. Their study concluded that HLA-DRB1*04 is the major restriction molecule for Fy^a-derived peptides suggesting that stimulation can cause an individual to produce the antibody [18]. Similarly, Raos et al associated HLA-DR and HLA-DQ polymorphisms with alloimmunization to the Fy^a antigen in a Croatian population [19].

Several studies have associated variants of HLA Class II with RBC alloimmunization and whether the inheritance of HLA Class II

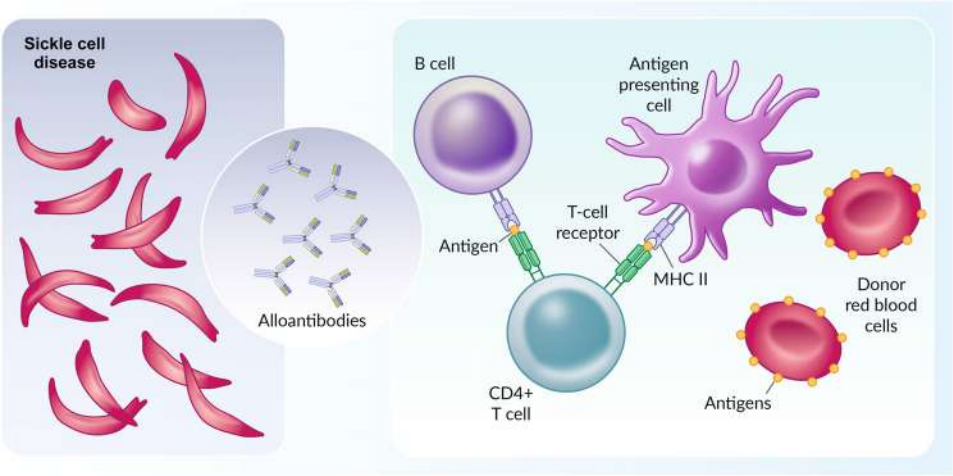

FIGURE 1 Red cell alloimmunization in sickle cell disease patients after exposure to donor cells' antigens

phenotypes provides susceptibility or protection against the formation of RBC alloantibodies [5, 7, 15, 18–26]. However, only a handful of these studies focused on the SCD population [5, 7, 15, 23, 24]. Many studies associated RBC alloimmunization with HLA-DRB1 variants indicating the role of HLA restriction in susceptibility to forming RBC antibodies, particularly in multi-responders. Maluskova et al demonstrated the association of HLA-DRB1*15 with multiple antibody responsiveness in relation to Rh phenotypes [25]. This supported the hypothesis that certain HLA polymorphisms could further increase susceptibility to multi-responsiveness towards RBC antigens.

Scope of review

Alloimmunization or the development of RBC alloantibodies is a major complication of blood transfusion, especially in chronically transfused SCD patients. Susceptibility to alloimmunization is dependent on several different factors, possibly leading to delayed HTRs (DHTR) or other adverse effects. However, not all patients exposed to mismatched RBC antigens become alloimmunized as only a minority of individuals form alloantibodies from exposure [27]. The differences in susceptibility have not yet been fully understood. As SCD patients, homozygous for the disease are multiply transfused, the exposure to different antigens increases the risk of alloimmunization. Some studies have associated HLA-DRB1 with RBC alloimmunization risks. HLA Class II alleles could potentially be a risk factor for developing RBC alloimmunization upon exposure to foreign antigens.

This study evaluates the presence of HLA Class II alleles in alloimmunized and non-alloimmunized SCD individuals homozygous for the sickling phenotype. The primary aim of this study is to investigate the association between HLA phenotype and its correlation to RBC alloimmunization susceptibility within the multiply transfused SCD population. Second, to identify the HLA Class II alleles that can cause an individual with SCD to be more susceptible to forming RBC antibodies after transfusion.

METHODS

Study design

A systematic review and meta-analysis were conducted to investigate the association between HLA Class II polymorphisms resulting in alloimmunization. This study focused on the increased risk of susceptibility to RBC alloimmunization in SCD patients with HLA Class II genetic polymorphisms.

Literature search strategy

For identifying literature for the systematic review and meta-analysis, the study followed the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-analysis) guidelines [28]. A systematic search on the PubMed, Scopus, Ovid and Google Scholar databases was conducted between the years 2000–2021, inclusive. The keywords ("HLA" OR "Human leukocyte antigen" OR "MHC") AND ("Sickle cell disease" OR "Sickle cell anaemia" OR "SCD" OR "haemoglobinopathies") AND "Alloimmunization" were used in conjunction during the searches. MeSH terms for variations in "HLA" and "sickle cell disease" were included to broaden the search. Furthermore, a manual search for additional literature was conducted. All articles retrieved underwent an eligibility criterion.

Inclusion and exclusion criteria

The PICO strategy (Population, Intervention, Comparison, Outcome) [29] was adopted for the study selection process to determine a generalized eligibility for inclusion of studies. Population: patients with SCD (homozygous for the HbSS genotype); Intervention: the HLA Class II allelic polymorphisms; Comparison: non-alloimmunized individuals; Outcome: resulting in the alloimmunization of red blood cells (RBCs) confirmed by serological or molecular techniques. The criteria

did not have a restriction on sample size or specific HLA Class II polymorphic gene. However, the inclusion criteria required (a) original articles, (b) the development of RBC alloimmunization associated with HLA polymorphism, and (c) a sample of patients with SCD (homozygous HbSS). Exclusion criteria were all articles that were (a) not in English, (b) duplicates, (c) other systemic reviews, (d) those unrelated to HLA or alloimmunization of RBCs or SCD, and (e) full texts that were unavailable.

Data extraction

The information obtained from the literature included the following information: author, year, sample size, objectives, design characteristic (prospective, retrospective), patient population, country of study implementation, HLA allele identified and methods used. Each study was assessed using a STROBE (Strengthening the Reporting Observational studies in Epidemiology) checklist [30] to investigate the suitability for the meta-analysis.

Statistical analysis

Data collected from the studies compared the number of alloimmunized and non-alloimmunized SCD patients with the presence of HLA-DRB1*04, HLA-DRB1*15 or HLA-DQB1*03. The association between increased susceptibility to alloimmunization and HLA Class II polymorphisms was assessed using odds ratios (ORs) with 95% confidence intervals (95% CI).

From the selected studies, the binary random-effect method utilizing the maximum likelihood model approach was selected to estimate the effect size as an odds ratio with 95% CI and heterogeneity. Significance was determined using p-value (where $p \leq 0.05$ is considered statistically significant). The heterogeneity of the studies using the chi-square-based Q test and I^2 test relative to the degree of freedom was evaluated (where $p \leq 0.05$ is considered statistically significant). Thresholds for the interpretation of heterogeneity follows: (i) 0%–40% might not be important, (ii) 30%–60% may represent moderate heterogeneity, (iii) 50%–90% may represent substantial heterogeneity, and (iv) 75%–100% considerable heterogeneity [31].

All statistical analyses were conducted using the Review Manager (RevMan) software (Version 5.4. The Cochrane Collaboration, 2020.) to evaluate the association between SCD patients and HLA alleles relative to RBC alloimmunization.

RESULTS

Study selection and characteristics of the included studies

Between the years 2000 and 2021, a total of 866 articles were collected from the four databases, with an additional 17 articles manually found. Of these 883 articles, 850 articles were excluded, as described in Figure 2. The remaining 33 articles were eligible for the systematic review. Among the 33 eligible articles, only four of these presented an analysis of HLA Class II alleles in SCD patients causing susceptibility towards alloimmunization and were included in the meta-analysis.

The four eligible studies presented analyses of HLA-DRB1 and/or HLA-DQB1 alleles within the SCD population. Sufficient data on HLA-DRB1 analysis in association with generalized RBC alloimmunization could be found in three studies [7, 23, 24], and one study [15] contained data on HLA-DQB1 in association with alloimmunization susceptibility. Table 1 summarizes the four studies included in the systematic review and meta-analysis. All studies were either conducted in the USA ($n = 2$) or Brazil ($n = 2$), consisting of all SCD patients with the homozygous genotype. All patients had a history of transfusion, and data on alloimmunization and non-alloimmunization were collected by each study. The studies varied in the HLA Class II allele found to be associated with alloimmunization susceptibility between HLA-DRB1*15, HLA-DRB1*04 and HLA-DQB1*03. The study conducted by Rodrigues et al [15], associated HLA-DRB1*04 and HLA-DRB1*11 to susceptibility but more specificity to producing anti-Fy^a and anti-K antibodies. HLA detection was consistent among all studies using polymerase chain reaction-sequence specific oligonucleotide (PCR-SSO). The method of alloimmunization detection varied; however, the primary endpoint was any RBC alloimmunization in all cases.

Quality assessment of studies

The quality of the four eligible studies was analysed and assessed using the STROBE standards for the meta-analysis. Table 2 addresses the most important points within each area of the studies conducted, according to the author. The following criterion included were determined from (1) introduction, (2) methods, (3) results, and (4) discussion. Within these four criterion areas, the quality of the studies was based on (1a) rationale explained, (1b) objectives specified with a hypothesis, (2a) patient eligibility provided, (2b) outcomes and potential confounders defined, (2c) bias addressed, (2d) describes all statistical methods, (3a) number of individuals at each stage reported, (3b) give adjusted estimates, and (4a) states limitations. The quality of each study for criteria was assessed as either "yes" or "no".

Patient eligibility was reported in three of the four studies, describing the sample size and number of transfusion histories required for data inclusion. The fourth study [7], however, did not clearly state the patient eligibility but did state the number of both alloimmunized and non-alloimmunized patients. In assessing the studies, only the study conducted by Sippert et al contained a healthy control group of experimental SCD patients. These healthy control patients with a similar ethnic background to the experimental groups served as the basis for allele frequency of polymorphism determination. One study [23] failed to define potential confounders in the study. Another study [23] failed to state any limitations. Overall, the bias of the primary studies was not well addressed. The transfusion histories of the patients for the studies were obtained from

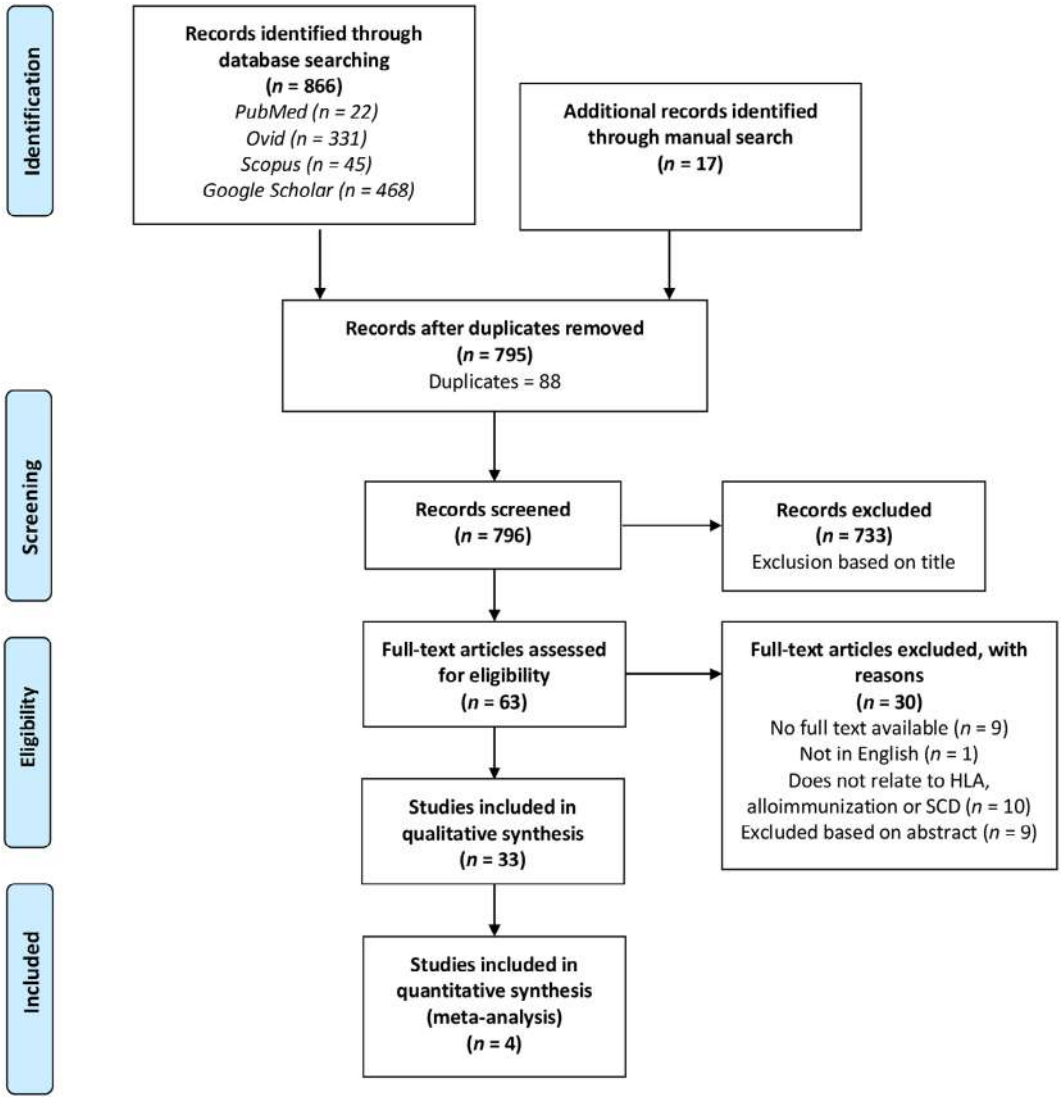

FIGURE 2 Flow diagram summary of a study selection process involving four databases (PubMed, Ovid, Scopus and Google scholar). A manual search was conducted in conjunction with 88 article duplications removed. Four major steps in the study selection process involve the general identification of articles, a screening process to exclude articles based on titles, followed by an eligibility check and finally, studies to be included in the meta-analysis based on relevant quantitative data [28]

institutional blood and medical records [7]; the University of Campinas (UNICAMP) Haematology Blood Center and Maringa Regional Blood Center [15]; the Hematology and Hemotherapy Center of the UNICAMP [23]; and the Children's National Medical Center [24].

Data analysis association

All included studies demonstrated that SCD patients have an increased risk of alloimmunization compared to the control group. The pooled results indicated a statistically significant association (OR = 2.33; 95% CI, 1.58–3.44; $p \leq 0.0001$). The heterogeneity between the studies was not important ($I^2 = 0\%$; $p = 0.88$) (Figure 3).

Table 3 summarizes the results from the four studies of SCD patients associated with increased susceptibility to RBC antibody production. The frequency of the alloimmunized SCD patients is greater than the non-alloimmunized patients. Figure 4 summarizes the frequency of HLA alleles that is associated with increased susceptibility to RBC antibody production in each of the included studies; however, the overall effect was not statistically significant (OR = 1.32; 95% CI, 0.76–2.30; $p = 0.32$).

Analysis of HLA Class II and alloimmunization/ strength of association

The majority of the studies addressing HLA polymorphism in association with generalized RBC alloimmunization reported on HLA-DRB1 alleles. Common antibodies reported in the studied alloimmunized patients included antibodies developed against the Rh system (anti-E,

TABLE 1 Summary of studies included in the meta-analysis on HLA Class II associated with RBC alloimmunization in SCD patients

Study	Study design	Sample size	Number of alloantibody positive & negative	Country	Population (nationality)	Method of HLA detection	Method of alloimmunization detection	HLA Class II associated with susceptibility
Hoppe C. [7]	Retrospective	159	+ve = 59 −ve = 100	USA	SCD (HbSS mutation) – American	PCR-SSO(Probe)	Standard (gel and antiglobulin techniques)	HLA-DRB1*15 & HLA-DRB1-11, HLA-DRB1*13
Rodrigues C. [15]	Prospective	172	+ve = 44 −ve = 128	Brazil	SCD (HbSS mutation) – Brazilian (mixed ethnic group)	PCR-SSO	Blood group genotyping by DNA microarray	HLA-DQB1*03 & HLA-DRB1*04, HLA-DRB1*11
Sippert E. [23]	Prospective	161	+ve = 67 −ve = 94	Brazil	SCD (HbSS mutation) -Brazilian	PCR-SSO	Serologic and molecular (Immucor BioArray Beadchip)	HLA-DRB1*15
Tatari-Calderone Z. [24]	Retrospective	204	+ve = 88 −ve = 116	USA	SCD (HbSS mutation) – African American	PCR-SSO using IMGT database	N/A	HLA-DRB*04

Abbreviations: +ve, positive; −ve, negative; HbSS, haemoglobin SS; SCD, sickle cell disease.

TABLE 2 STROBE checklist for studies used in meta-analysis identified across the different areas of a study. Using a series of criteria to assess the relevance and quality of studies

	Introduction		Methods				Results		Discussion
Study	Rationale explained	Objectives specified with hypothesis	Patient eligibility provided	Outcomes, and potential confounders defined	Bias addressed	Describes all statistical methods	Number of individuals at each stage reported	Give adjusted estimates (95% CI)	States limitations
Hoppe C. [7]	Y	Y	N	N	Y	Y	Y	Y	Y
Rodrigues C. [15]	Y	Y	Y	Y	N	Y	Y	Y	N
Sippert E. [23]	Y	Y	Y	N	N	Y	Y	Y	Y
Tatari-Calderone Z. [24]	Y	Y	Y	Y	Y	Y	Y	Y	Y

Abbreviations: 95% CI, 95% confidence interval; N, No; Y, Yes.

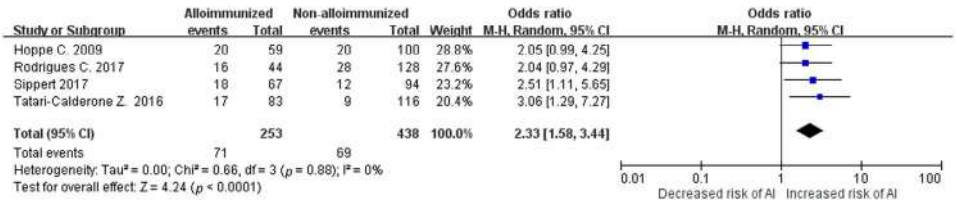

FIGURE 3 Forest plot of the risk of alloimmunization in SCD patients. Results were expressed as the odds ratio (OR) with 95% confidence intervals (CI), the random-effect model (DerSimonian and Laird method) was used for the overall effect, and mantel–Haenszel method (M-H) was used for the heterogeneity. Statistical significance of the forest plot was expressed as Z-score and *p*-value; heterogeneity was described as I^2 with a different *p*-value. The weight was defined as % regarding the size of each study

TABLE 3 Summary of studies reporting number and frequency of alloimmunized and non-alloimmunized SCD patients

Study	Red cell units chosen for the patients	Number of alloimmunized/total alloimmunized	Alloimmunized frequency (%)	Number of non-alloimmunized/total alloimmunized	Non-alloimmunized frequency (%)	OR (95% CI)
Hoppe C. [7]	At least Rh and Kell matched	20/59	34.00	20/100	20.00	2.05 (0.99, 4.25)
Rodrigues C. [15]	At least Rh and Kell matched	31/44	35.23	55/128	21.48	2.04 (0.97, 4.29)
Sippert E. [23]	At least Rh and Kell matched	18/67	13.85	12/94	6.98	2.51 (1.12, 5.65)
Tatari-Calderone Z. [24]	At least Rh and Kell matched	17/88	19.31	9/116	7.76	2.85 (1.20, 6.74)

Abbreviations: 95% CI, 95% confidence interval; OR, odds ratio.

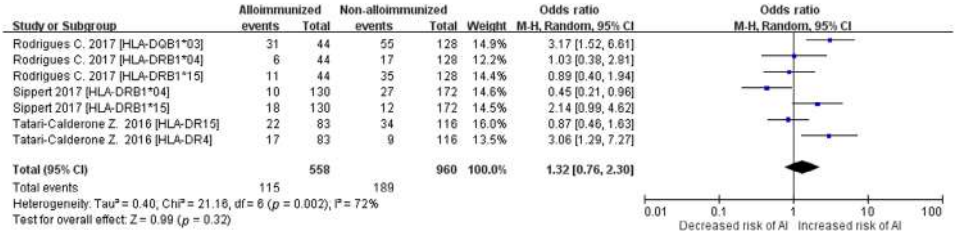

FIGURE 4 Forest plot of the frequency of HLA alleles that are associated with increased susceptibility to RBC antibody production in each included study. Results were expressed as the odds ratio (OR) with 95% confidence intervals (CI), the random-effect model (DerSimonian and Laird method) was used for the overall effect, and mantel–Haenszel method (M-H) was used for the heterogeneity. Statistical significance of the forest plot was expressed as Z-score and *p*-value; heterogeneity was described as I^2 with a different *p*-value. The weight was defined as % regarding the size of each study

TABLE 4 Strength of association of HLA Class II alleles to alloimmunization rate

Study	HLA phenotype	RBC antibody	Alloimmunized (ab present)	Non-alloimmunized (ab absent)
Hoppe C. [7]	HLA-DRB1*04 HLA-DRB1*11	Anti-Fy[a] Anti-K	100% 47%	12% 19%
Rodrigues C. [15]	HLA-DRB1*11 & -DRB1*13	Anti-K	70%	50%
Sippert E. [23]	HLA*DRB1*15	Rh system (D,C,c,E,e)	15.63%	6.98%
Tatari-Calderone Z. [24]	HLA-DRB1*04	Anti-Fy[a]	100%	19.01%

Abbreviations: Ab, antibody; HLA, human leukocyte antigen; RBC, red blood cell.

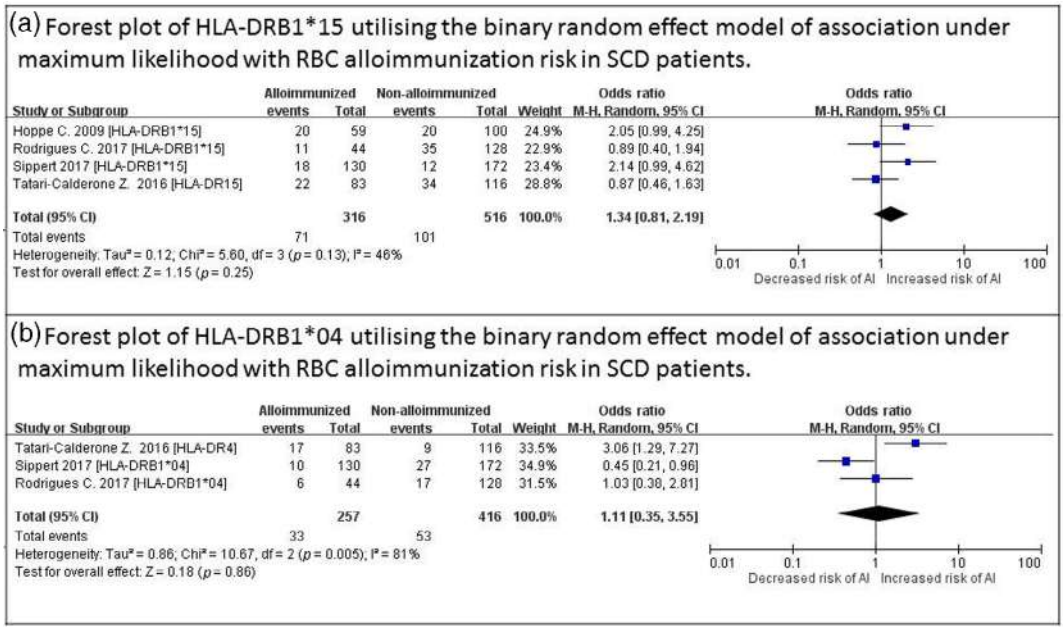

FIGURE 5 (a) Forest plot of HLA-DRB1*15 utilizing the binary random-effect model of association under maximum likelihood with RBC alloimmunization risk in SCD patients. (b) Forest plot of HLA-DRB1*15 utilizing the binary random-effect model of association under maximum likelihood with RBC alloimmunization risk in SCD patients. Results were expressed as the odds ratio (OR) with 95% confidence intervals (CI), the random-effect model (DerSimonian and Laird method) was used for the overall effect, and mantel–Haenszel method (M-H) was used for the heterogeneity. Statistical significance of the forest plot was expressed as Z-score and *p*-value; heterogeneity was described as I^2 with a different *p*-value. The weight was defined as % regarding the size of each study

anti-C, anti-D, anti-C^w, anti-c and anti-e) and the Kell system (anti-K). Other antibodies less frequently reported included anti-Fy^a, anti-S, anti-Jk^b, anti-Le^a, anti-M, anti-I and more [7, 15, 23, 24].

The studies included have associated some HLA Class II alleles with specific alloantibody formation as seen in Table 4. In the study conducted by Rodrigues et al, an increased prevalence of HLA-DRB1*04 was observed in anti-Fy^a patients (100%) in comparison to the alloantibody negative control patients (12%). Rodrigues et al have also demonstrated the predominance of the HLA-DRB1*11 allele in patients with anti-K (47%) compared to those without anti-K (19%) [15]. Similarly, Sippert et al reported a prevalence of the HLA-DRB1*15 allele in patients alloimmunized to the Rh system antigens (D, C, E, c, e) (15.63%), compared to the non-alloimmunized controls (6.98%) [23]. Furthermore, Hoppe et al found an excess of HLA-DRB1*11 and -DRB1*13 alleles in anti-K patients (70%) when compared to the controls (50%) [7]. Tatari-Calderone et al found that the HLA-DRB1*04 molecule acts as a restriction molecule in the development of anti-Fy^a antibodies in the Caucasian population. They demonstrated that 100% of individuals expressing the -DRB1*04 phenotype with anti-Fy^a compared to 19.01% non-alloimmunized individuals carrying the -DRB1*04 phenotype. However, Tatari-Calderone et al, reported a conflicting statement to Rodrigues and coworkers that HLA-DQB1*03 conveyed a protective role towards RBC alloimmunization [24].

Four studies were included in the meta-analysis for the association between HLA-DRB1*15. However, due to the varying results from each study, the result was not statistically significant (OR, 1.34; 95% CI, 0.81–2.19; $I^2 = 46\%$; $p = 0.25$) as demonstrated in Figure 5a. Moreover, three studies were included in the meta-analysis for the association between HLA DRB1*04, but the result was not statistically significant due to the limited number of studies included and the result variation (OR, 1.11; 95% CI, 0.35–3.55; $I^2 = 81\%$; $p = 0.86$) as illustrated in Figure 5b.

DISCUSSION

SCD individuals require many transfusions in their lifetimes for related complications to the sickling disease. Factors increasing the risk of RBC alloimmunization in SCD patients remains largely unclear, however, genetic factors are known to influence immune regulatory responses. The risk of alloimmunization is related to the number of red cell transfusions (transfusion intensity) and the degree of prophylactic antigen matching. Alloimmunization is dependent on the presentation of donor RBC-related peptide antigens by APCs to the recipient's T-cell receptors. Therefore, HLA Class II molecules are essential for RBC alloimmunization. A decrease in alloimmunization rates has been shown in SCD patients transfused with RBCs matched for C/c, E/e and K antigens in multiple single-group studies by the ASH 2020 guidelines for SCD transfusion support [32]. This demonstrated that SCD patients receiving Rh and K-matched red cells showed an alloimmunization risk of 18% (95% CI, 10%–27%) acquired from 15 studies. However, despite prophylactic measures to ensure more compatible units are provided, RBC alloimmunization remains

highly prevalent in multiply transfused SCD patients. Many SCD individuals are still developing antibodies to the Rh system antigens despite the serological matching of units. This may be due to allelic polymorphisms within the blood group systems, mainly the Rh system, off-protocol transfusion, immune hyper-responsiveness during proinflammatory events or other unidentified causes [33].

The current systemic review and meta-analysis examined the association of HLA polymorphisms and RBC alloimmunization in SCD patients from Brazil and the United States. The review and meta-analysis have confirmed that the genetic inheritance of some HLA alleles predisposes individuals to RBC alloimmunization. These observations led to the findings that antibody production is not only caused by chronic transfusions but also by genetic components influencing the development of alloantibodies [34].

Although all studies selected the minimum Rh and Kell matched RBC units for the patients, most of the antibodies identified in the alloimmunized groups were in the Rh and Kell systems. While some alloimmunization may be due to polymorphisms in the RH and Kell system genes, it is likely that prophylactic matching was incompletely practised. Between the articles used in this review, there are some contradictions in relation to which alleles cause alloimmunization susceptibility and to which blood group antigens.

Interpretations

The overall result of the present study indicates the odds of SCD patients developing RBC alloantibodies are greater than non-SCD patients after antigenic exposure. An OR of 2.33 demonstrates statistically significant evidence that there is an increased odds of developing alloantibodies if an individual is an SCD patient. The heterogeneity demonstrated no importance and thus, indicates that the differences between the studies are due to random sampling error. This assures that genetic inheritance of HLA Class II alleles producing alloimmunization risk will cause similar effects in other multiply transfused populations. These results are supported by studies relating HLA Class II alleles, mainly HLA-DRB1 polymorphisms, to specific alloantibody development in the polytransfused population.

The primary studies from this review identified associations of HLA Class II alleles with anti-Fy^a, anti-K and antibodies to the Rh system antigens. Evidence towards alloimmunization with anti-Fy^a showed a strong relationship with the HLA-DRB1*04 allele [15, 24]. The majority of SCD patients are of African descent, where many have the Fy(a-b-) phenotype mostly due to the GATA mutation [35]. Although these individuals are serologically negative for Fy^b on their RBCs, other cells will, however, express Fy^b. This means that there is a low risk of developing an antibody to the Fy^b antigen [35]. As such, it possibly explains why alloimmunization risk is not associated with anti-Fy^b. Furthermore, a moderately strong association with anti-K alloimmunization was found with mainly HLA-DRB1*11; however, antibodies to the Rh system antigens were not as convincing when associated with HLA-DRB1*15.

Many other studies have investigated the role HLA alleles play in alloimmunization susceptibility of other populations, which reinforces the findings of this current review. In a study by Reviron et al [22], HLA-DRB1*01 was found significantly more frequent in patients with anti-Jk^a than the controls, 55%–17%, respectively [22]. Similarly, Baleotti et al [21] had concluded that there was an overrepresentation of HLA-DRB1*07 in patients alloimmunized with anti-Di^a compared to non-alloimmunized patients, 75% and 27%, respectively. Furthermore, HLA-DRB1*11 demonstrated by Chiaroni et al [20] associated with K immunization was significantly higher in alloimmunized patients compared to controls (57% vs. 28%, respectively); this was confirmed by Rodrigues et al. In confliction to Chiaroni and colleagues and Rodrigues and colleagues, Noizat-Pirenne et al [18] identified several HLA-DRB1 groups that were seen increasingly in anti-K individuals. Furthermore, HLA-DRB1*15 has been shown to be involved with alloimmunization by the D and Fy^a antigens [19, 23, 36]. Raos et al and Picard et al had also found an overrepresentation of HLA-DRB1*04 and/or HLA-DRB1*15 alleles in anti-Fy^a patients in the Croatian and Southern European population, respectively (96% for both) [19, 37].

Due to linkage disequilibrium between alleles across the major histocompatibility complex (MHC), it is difficult to determine the direct response that an HLA phenotype has in association with RBC alloimmunization [23]. Despite this, the results have demonstrated that HLA Class II alleles convey susceptibility to RBC alloimmunization, especially HLA-DRB1, supported by several studies across different populations.

Implications to clinical practice

Due to the presence of alloantibodies in chronically transfused SCD patients that cause HTRs and other transfusion-related complications making it difficult to obtain compatible blood units, it is important to investigate and identify the association between HLA and alloimmunization. This will allow for the best selection of compatible, phenotype-matched units for patients. Rodrigues and colleagues [15] investigated the possibility of molecular techniques in routine laboratory immunohaematology to increase the safety and efficacy of transfusion. SCD is most commonly found in patients of African descent, showing a high rate of complex Rh antibody variants and antibodies to low-incident antigens [38]. Hendrickson and colleagues [37] concluded that antigen matching based on phenotype alone between African American donors to African American SCD recipients may not decrease alloimmunization rates to complex Rh antibody variants. Therefore, making genotyping RBC antigens for matched units with a beneficial alternative.

Overall, a decrease in alloimmunization rates among sickle cell patients has been demonstrated with the use of genotyping. A Brazilian study found that 11 of 15 alloimmunized patients benefited from receiving RBC units based on genotype [39]. However, due to limited blood supply, molecular matching may not be a feasible option as it is also a costly procedure. Such a precise matching practice

makes routine transfusion difficult for both the transfusion service and donor centre and is, therefore, not widely applied.

Strengths and limitations

This present review systematically examines the association between HLA Class II and RBC alloimmunization with respect to HLA-DRB1 and -DQB1 alleles. Despite the many ways to attain alloimmunization, these factors did not seem to affect the results. The review defines a more specific subgroup of chronically transfused patients, SCD individuals.

However, this systematic review is unable to conclude the association between specific alleles and the risk of alloimmunization due to the limited number of studies incorporated. The included studies show different HLA associations, and the overall results are not statistically significant (Figure 5a,b).

Future studies should include a larger sample size with a healthy control group. More extensive investigation in SCD patients would need to be conducted as there are still some discrepancies between the alleles conveying susceptibility. Thus far, convincing evidence towards HLA-DRB1*04 resulting in anti-Fy^a has been illustrated. However, more is required to identify which SCD patients are responders or non-responders based on homozygosity, heterozygosity or other factors that may play a role.

This study could not account for other factors, including age, gender, and the number of previous transfusions, which may impact results. Additionally, homozygosity and heterozygosity of alleles were not considered, such as a haplotype with alleles considered as protective and susceptible. Moreover, the studies did not directly compare the difference between serologic and genotypic matching for the prevention of RBC alloimmunization. All primary studies determined the alloimmunization outcome; however, not all studies assessed the risk of bias. Rodrigues et al and Sippert et al corrected the data with Bonferroni's method [15, 23].

It is not well known why SCD patients given antigen-negative units could develop new alloantibodies. A further study focusing on the association between HLA and RBC alloimmunization in SCD patients will be highly beneficial for healthcare professionals. It could provide clinicians and transfusion services with a better understanding of the risk of multiple transfusions in SCD patients and introduce a suitable strategy to prevent the development of alloantibodies.

CONCLUSION

There are routine screening and crossmatching of blood in place to identify and prevent as many incompatibilities as possible. However, due to the requirement for more than one transfusion and the lack of antigen-negative units, there is a high risk for SCD patients to become alloimmunized. In conclusion, the result from this study suggests that there is no statistically significant evidence indicating that HLA Class II alleles are associated with susceptibility to alloimmunization in multiply transfused SCD patients. The reasons are unknown as to how HLA alleles impact alloimmunization susceptibility. Further investigation should be conducted into the GATA mutation and whether there is a susceptibility or risk factor towards RBC alloimmunization. Furthermore, while genetic variants are not picked up in routine screening, further study is needed into the use of genotyping for extended matching in SCD patients as a preventative measure to alloantibody formation. Heterozygosity and/or homozygosity inheritance of the alleles should be investigated for the same susceptibility effects. This knowledge could help change the screening process for HLA in SCD patients to better the transfusion system. Early identification of genetic factors affiliated with likely alloantibody development would allow for selective use of phenotypically matched RBC units in genetically susceptible SCD patients. This could ultimately reduce the likelihood of RBC alloimmunization.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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ORIGINAL ARTICLE

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Explainable haemoglobin deferral predictions using machine learning models: Interpretation and consequences for the blood supply

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Abstract

Background and Objectives: Accurate predictions of haemoglobin (Hb) deferral for whole-blood donors could aid blood banks in reducing deferral rates and increasing efficiency and donor motivation. Complex models are needed to make accurate predictions, but predictions must also be explainable. Before the implementation of a prediction model, its impact on the blood supply should be estimated to avoid shortages.

Materials and Methods: Donation visits between October 2017 and December 2021 were selected from Sanquin's database system. The following variables were available for each visit: donor sex, age, donation start time, month, number of donations in the last 24 months, most recent ferritin level, days since last ferritin measurement, Hb at nth previous visit (n between 1 and 5), days since the nth previous visit. Outcome Hb deferral has two classes: deferred and not deferred. Support vector machines were used as prediction models, and SHapley Additive exPlanations values were used to quantify the contribution of each variable to the model predictions. Performance was assessed using precision and recall. The potential impact on blood supply was estimated by predicting deferral at earlier or later donation dates.

Results: We present a model that predicts Hb deferral in an explainable way. If used in practice, 64% of non-deferred donors would be invited on or before their original donation date, while 80% of deferred donors would be invited later.

Conclusion: By using this model to invite donors, the number of blood bank visits would increase by 15%, while deferral rates would decrease by 60% (currently 3% for women and 1% for men).

Keywords

blood donation testing, donor health, haemoglobin measurement

Highlights

- Support vector machines can provide explainable haemoglobin (Hb) deferral predictions that are biologically sensible.

- Using prediction models to guide donor invitations may increase blood bank visits and decrease donor deferral rates.
- Including a greater number of previous Hb measurements from the same donor increases prediction accuracy.

INTRODUCTION

Sanquin, the Dutch national blood service, collects over 400,000 whole-blood donations from non-remunerated, voluntary blood donors every year. Women may donate a maximum of three times per year, and men five times. Haemoglobin levels are tested before every donation to prevent blood collection from donors with insufficient iron. The minimum haemoglobin level for blood donation is 7.8 mmol/L for women and 8.4 mmol/L for men; if the capillary Hb-test (HemoCue) shows a lower value, the donor is deferred for 3 months, that is, sent home without donating blood. If the haemoglobin value is more than 0.5 mmol/L below the donation threshold, the donor is referred to a donor physician. Additionally, since October 2017, ferritin levels have been measured in each new donor, as well as after every fifth donation in repeat donors. Donors are deferred for 6 months if their ferritin level is between 15 and 30 ng/ml, or for 12 months if their ferritin level is below 15 ng/ml. This ferritin deferral policy was implemented because haemoglobin is a poor indicator of iron stores, as iron deficient donors can still present with sufficient haemoglobin levels until the iron deficiency is very severe.

While it is important to defer donors that do not meet donation requirements, sending donors home without giving them the opportunity to donate is discouraging and costly. Previous studies have shown that donors are less likely to return to the blood bank after a deferral for low haemoglobin than after a successful donation, especially if it concerns their first blood bank visit [1]. This is less likely after deferral for low ferritin levels, which occurs by letter after the donation, indicating that post-donation deferral is less demotivating for donors than on-site deferral [2]. The implementation of ferritin testing has had a considerable impact on the blood supply, as a large part of the existing donor population (53% of women and 42% of men) were found to have ferritin levels below 30 ng/ml and had to be deferred [3]. However, this has had the intended positive impact on donor deferral rates due to low haemoglobin, which decreased from 8% for women and 3% for men in 2016 to 3% for women and 1% for men in 2021 [4].

Although percentage-wise, haemoglobin deferral rates are quite low in the Netherlands, they still amount to about 8000 deferrals each year, and there is a risk of permanently losing these donors. To reduce deferral rates and improve donor motivation, we should re-think haemoglobin deferral policies. One tool that can be used for this purpose is a haemoglobin deferral prediction model. Many of these prediction models have already been developed, including models that predict personalized donation intervals [5–7]. Prediction models can be used in the donor invitation process by predicting haemoglobin deferral for eligible donors and only inviting those donors that are predicted to not be deferred. Because deferred donors are only a small proportion of the total donor population, it has proven difficult to accurately identify them, and hence prediction models are not used in practice yet.

We present a novel machine learning haemoglobin deferral prediction model based on donor characteristics and donation history. New in our approach is that we use SHapley Additive exPlanations [8] to explain how the model uses the variables in its predictions and relate these explanations to known physiological processes. This gives valuable insight into the associations that are learned by the model; if prediction models are to be used to make decisions in practice, the user must understand how the model makes these decisions. Moreover, we show the potential impact that prediction models can have on the total blood supply, if these are to be used to guide donor invitations, by calculating deferral probabilities at multiple time points for each donor. By both explaining the predictions and assessing the impact of the model on the blood supply, we remove two important limitations that currently prevent blood services from implementing prediction models.

METHODS

Data used

Data on blood bank visits by whole-blood donors were extracted from Sanquin's database system eProgesa, for donations. Only data from donors who explicitly provided informed consent for the use of their data for scientific research were used. This consent is given by more than 99% of all donors. For each visit, the following information was collected: donor sex, donor age, donation date, donation (registration) time, haemoglobin level and ferritin level. Ferritin is measured at every new donor intake and upon every fifth donation in repeat donors. Therefore, ferritin levels are unavailable for most donations. By using these data, predictor variables were calculated for each visit, as described in Table 1.

In total, 938,710 blood bank visits (excluding new donor intakes and donation types other than whole blood) by 241,131 unique donors were registered between October 2017 and December 2021. After excluding visits for which no previous ferritin measurement was available, 458,615 blood bank visits by 157,423 unique donors remained for the analysis.

The outcome variable 'HbOK' is dichotomous; deferral (Hb level below the eligibility threshold for donation) or non-deferral (Hb equal to or above the threshold).

TABLE 1 Predictor variables

Variable	Unit or values	Description
Sex	{male, female}	Biological sex of the donor; separate models are trained for men and women
Age	years	Donor age at time of donation
Time	hours	Registration time when the donor arrived at the blood bank
Month	{1–12}	Month of the year that the visit took place
NumDon	count	Number of successful (collected volume >250 ml) whole-blood donations in the last 24 months
FerritinPrev	ng/ml	Most recent ferritin level measured in this donor
DaysSinceFer	days	Time since this donor's last ferritin measurement
HbPrev*n*	mmol/L	Haemoglobin level at n^{th} previous visit, for *n* between 1 and 5
DaysSinceHb*n*	days	Time since related Hb measurement at n^{th} previous visit, for *n* between 1 and 5

Analyses

Support vector machines (SVMs) [9] are used to predict haemoglobin deferral. SVMs are supervised machine learning models that find the optimal hyperplane separating the outcome classes based on the predictor variables of a so-called *training set*. After fitting the model on the training set, the model can predict the outcome class of unseen observations called the *test set*. It also gives the probability of an observation belonging to each outcome class. We chose SVMs as a classification algorithm because all predictor variables are numeric, and it is computationally less expensive than, for instance, K-nearest neighbours or (dynamic) linear mixed models.

For each sex, five SVMs were trained, named SVM-*n* for *n* between one and five, indicating the number of previous blood bank visits (HbPrev*n* and DaysSinceHb*n*) used as predictor variables. Donors are only included in SVM-*n* if they have at least *n* previous visits; therefore, sample sizes decrease from SVM-1 to SVM-5. Blood bank visits before 2021 were used as the training set, while visits in 2021 were used as the test set to validate performance on unseen data. This division was chosen over a random training/test division because if these models were used in practice, they would be trained on all historical data and applied to future data. We used a paired t-test to assess the difference in deferral rates between training and test sets of donors of the same sex with the same number of previous donations. To assess the generalizability of the model to new donors, we did a separate experiment in which the test set is comprised of the last blood bank visit of 20% of all unique donors, and the training set includes all donations from the remaining 80% of donors.

TABLE 2 Interpretation of performance metrics

Metric	Outcome class	Definition
Precision	Deferral	The proportion of donations correctly classified as deferrals by the model, out of all donations classified as deferrals.
Recall	Deferral	The proportion of donations correctly classified as deferrals by the model, out of all donations classified as true deferrals.
Precision	Non-deferral	The proportion of true non-deferrals, out of all predicted non-deferrals.
Recall	Non-deferral	The proportion of predicted non-deferrals, out of all true non-deferrals.

For each of the 10 models, that is, SVM-1 through SVM-5 for both sexes, hyperparameters were optimized separately, using stratified (on the outcome variable) five-fold cross-validation within the training set data (and thus not using the test data). Hyperparameters were optimized using grid search, using balanced accuracy as a scoring method, defined as the weighted average of recall in both classes (see Table 2 for the definition of recall). This method is especially suitable for imbalanced datasets because it uses class-balanced sample weights to determine the average recall.

Precision and recall were determined and compared for training and test datasets for each model. Both metrics are calculated for both outcome classes. A practical interpretation of these metrics is given in Table 2.

To explain the model predictions, we used SHapley Additive exPlanations (SHAP) values, a model agnostic explainer. SHAP values show the contribution of each variable to the prediction for each individual observation, which is even more informative than coefficients returned by, for example, linear models. By summarizing observation-based contributions, we obtain variable importance measures for a model that does not have interpretable coefficients.

Potential impact on the blood supply

We assessed the potential impact of using SVMs to guide donor invitations by predicting deferral for all blood bank visits that took place in 2021 (the test set). For each observation, we used information of all previous blood bank visits (up to five) available as predictor variables. This means that SVM-1 is used when only one previous visit is available, SVM-2 if there are two previous visits, etc.

If prediction models are to be used in practice, they should estimate the deferral probability for different days in the future and invite a donor for the first occurrence where the non-deferral probability would exceed a preset value. To simulate this, we predicted haemoglobin deferral each week from 1 year before the original donation date to 1 year after, by adjusting all time-related variables. If the

predicted donation interval were to be less than the minimum donation interval (57 days for men, 122 days for women), the latter would be applied.

We compare all original donation intervals with the donation intervals as proposed by the model. Dividing the sum of the original donation intervals by the sum of the model-guided donation intervals

TABLE 3 Size of training and test datasets per model

	Training		Test	
Model	**Women**	**Men**	**Women**	**Men**
SVM-1	128,173 (4084; 3.19%)	121,746 (1339; 1.10%)	110,372 (3696; 3.35%)	98,324 (1074; 1.09%)
SVM-2	83,532 (2884; 3.45%)	96,441 (1133; 1.17%))	85,131 (3065; 3.60%)	84,000 (984; 1.17%)
SVM-3	59,720 (2032; 3.40%)	79,690 (997; 1.25%)	67,167 (2451; 3.65%)	72,576 (902; 1.24%)
SVM-4	47,317 (1494; 3.16%)	67,934 (887; 1.31%)	54,090 (1874; 3.46%)	63,447 (806; 1.27%)
SVM-5	40,604 (1113; 2.74%)	59,611 (768; 1.29%)	45,208 (1378; 3.05%)	55,582 (699; 1.26%)

Note: The number and percentage of deferrals is given in brackets.

TABLE 4 Marginal distributions of predictor variables, represented by median and interquartile range

Women					
Variable	**≥1 previous visit**	**≥2 previous visits**	**≥3 previous visits**	**≥4 previous visits**	**≥5 previous visits**
Age	30 (23–47)	32 (24–48)	34 (25–50)	35 (26–51)	36 (37–52)
NumDon	1 (0–3)	2 (1–3)	3 (2–4)	3 (2–4)	3 (3–4)
FerritinPrev	47 (33–74)	46 (33–70)	44 (32–65)	41 (31–59)	39 (29–55)
DaysSinceFer	237 (125–420)	329 (197–497)	383 (260–547)	400 (230–572)	372 (204–567)
HbPrev1	8.5 (8.1–8.9)	8.5 (8.1–8.9)	8.5 (8.1–8.9)	8.5 (8.1–8.9)	8.5 (8.1–8.9)
DaysSincePrev1	135 (105–196)	154 (132–211)	158 (132–217)	167 (133–224)	173 (133–236)
HbPrev2		8.5 (8.1–8.9)	8.5 (8.1–8.9)	8.5 (8.1–8.8)	8.5 (8.1–8.8)
DaysSincePrev2		302 (255–412)	328 (271–445)	336 (273–468)	349 (280–493)
HbPrev3			8.5 (8.1–8.8)	8.4 (8.1–8.8)	8.4 (8.1–8.8)
DaysSincePrev3			482 (398–644)	511 (420–674)	528 (430–696)
HbPrev4				8.4 (8.1–8.8)	8.4 (8.1–8.8)
DaysSincePrev4				674 (553–871)	709 (581–904)
HbPrev5					8.4 (8.1–8.8)
DaysSincePrev5					877 (721–1107)
Men					
Variable	**≥1 previous visit**	**≥2 previous visits**	**≥3 previous visits**	**≥4 previous visits**	**≥5 previous visits**
Age	34 (26–48)	35 (27–49)	36 (27–50)	37 (28–51)	38 (28–51)
NumDon	3 (1–5)	4 (2–5)	4 (3–6)	5 (3–6)	5 (4–6)
FerritinPrev	77 (44–141)	66 (40–126)	57 (38–108)	52 (36–89)	47 (35–73)
DaysSinceFer	200 (100–335)	232 (151–365)	257 (177–378)	271 (186–385)	267 (173–387)
HbPrev1	9.4 (9.0–9.9)	9.4 (9.0–9.9)	9.4 (9.0–9.8)	9.4 (8.9–9.8)	9.4 (8.9–9.8)
DaysSincePrev1	81 (63–133)	90 (67–147)	92 (69–160)	98 (70–168)	105 (70–176)
HbPrev2		9.4 (9.0–9.8)	9.4 (9.0–9.8)	9.4 (8.9–9.8)	9.4 (8.9–9.8)
DaysSincePrev2		185 (128–287)	196 (147–302)	210 (153–315)	219 (158–330)
HbPrev3			9.4 (9.0–9.8)	9.4 (9.0–9.8)	9.4 (8.9–9.8)
DaysSincePrev3			302 (225–441)	322 (238–463)	335 (245–485)
HbPrev4				9.4 (8.9–9.8)	9.4 (8.9–9.8)
DaysSincePrev4				424 (315–600)	444 (330–620)
HbPrev5					9.4 (8.9–9.8)
DaysSincePrev5					552 (416–752)

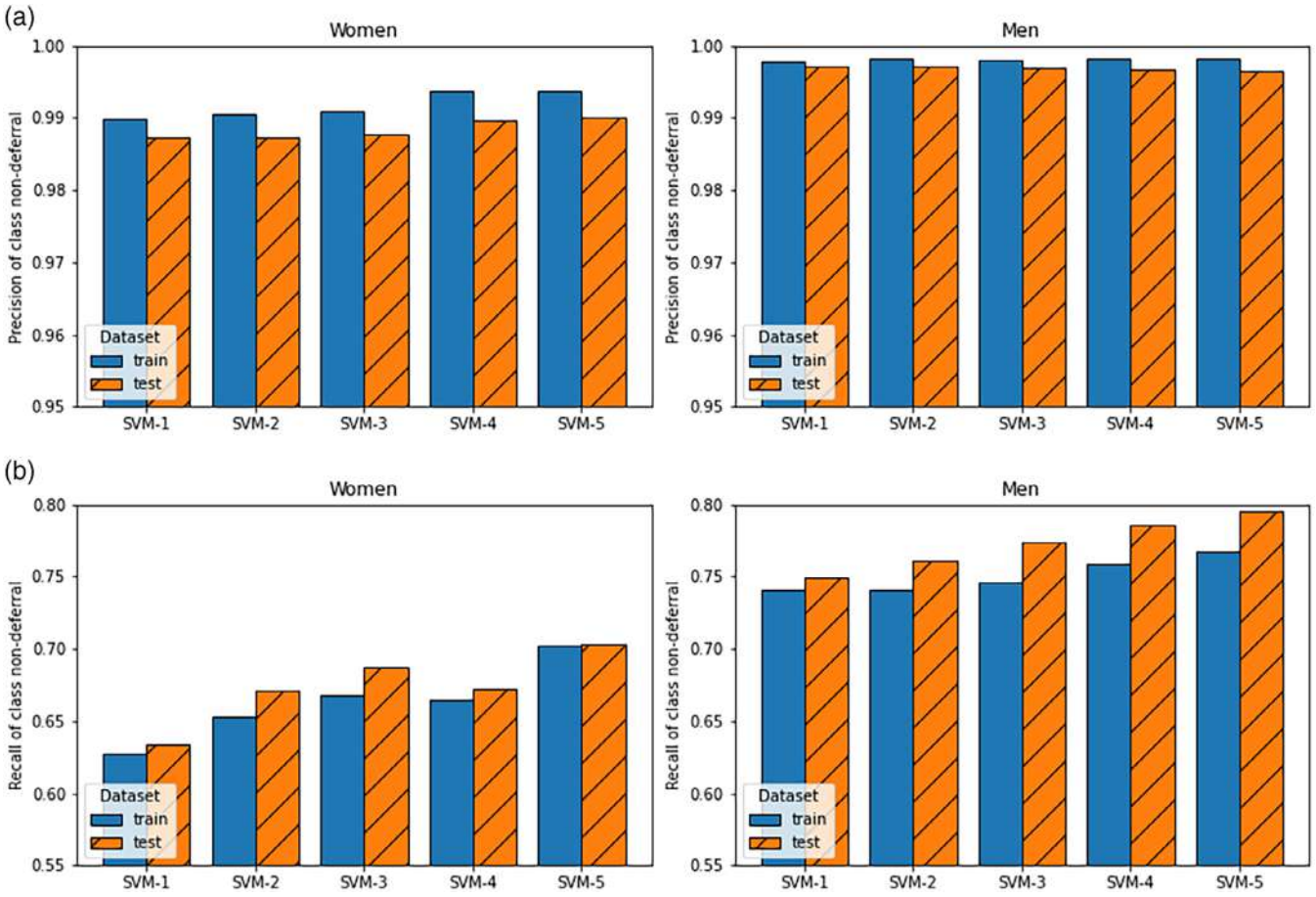

FIGURE 1 Performance metrics for all models (left: Women, right: Men). (a): Precision of class non-deferral; the proportion of successful donations among all predicted non-deferrals. The complement of the precision is the deferral rate, should the model be used to guide invitations. (b): Recall of class non-deferral; the proportion of successful donations that are predicted correctly. The complement of the recall is the proportion of missed donations, should the model be used to guide invitations. Note that the y-axes in are zoomed in to highlight the differences between various models

gives the relative change in blood bank visits per time unit and hence the relative yield of blood donations.

Software

All analyses were performed in Python 3.9, using modules *numpy* [10] and *pandas* [11] for data processing, *sklearn* [12] for model training and predictions, *shap* [8] for calculating SHAP values, and *matplotlib* [13] for creating graphs. The analysis code is available as a GitHub repository and indexed on Zenodo with https://doi.org/10.5281/zenodo.6938112.

RESULTS

Table 3 shows the sample sizes of training and test datasets for each model. Deferral rates in the training datasets are 3.19% (SD 0.28) for women and 1.22% (SD 0.09) for men; in the test sets, they are 3.42% (SD 0.24) for women and 1.21% (SD 0.08) for men. Using a paired t-test, the difference in deferral rate between the training and test datasets is significant for women ($p = 0.002$) but not for men ($p = 0.070$). No correction was made for the differing deferral rates, as the models are intended for future predictions, and in practice, the deferral rate of future blood bank visits is unknown. Also, a change in deferral rate should be correctly predicted by the model if the mechanism causing this change can be learned from the data. Deferral rates differ between models due to small differences in the data between subsets of the data (see Table 4). This is not a problem as long as the same associations between predictor variables and outcome are found in all subsets of the data, which is described in the feature importance part of the results.

Although the training datasets consist of 3 years of data, and the test datasets of only 1 year, their sizes are similar and sometimes the test dataset is even larger. This is because donations are only included from donors for whom at least one ferritin measurement was available. As ferritin screening was implemented using a stepped wedge approach (the first blood bank locations started in October 2017, but only in November 2019 all locations were included), the number of donors that could be included in the training dataset was limited [4].

Marginal distributions of predictor variables are described in Table 4. As the number of previous donations increases, the median age increases from 30 to 36 years for women and from 34 to 38 for men. The median values of the last ferritin measurement decreased from 47 ng/ml in SVM-1 to 39 ng/ml in SVM-5 for women and from 77 to 47 ng/ml for men. The median time between consecutive donations increases from SVM-1 to SVM-5, while previous haemoglobin levels are consistent across models, as well as different numbers of previous visits.

Accuracy and model fit

Figure 1 compares precision and recall for class non-deferral across all models. Performance on the training and test sets are similar, indicating that the models are well-fitted. Both precision and recall increase as more previous blood bank visits are used to make predictions. Re-running all models only on donors with at least five previous blood bank visits did not change this observed increase in performance. The models handle the difference between the proportions of deferral in the training and test set very well: comparing the observed difference in deferral proportion in the training and test set to the predicted difference, the mean difference of these differences is only 0.05 percentage points (maximum: 0.12 percentage points). This indicates that the models are robust against (modest) changes in deferral rates.

TABLE 5 Precision and recall for outcome class non-deferral, compared between two different training/test splits

Sex	Metric	Time split	Random split	Difference
Female	Precision	0.991	0.994	−0.003
	Recall	0.698	0.701	−0.003
Male	Precision	0.997	0.996	+0.001
	Recall	0.804	0.791	+0.013

Note: Results are shown for the test set of SVM-5. For the time split, the test set consists of donors whose previous donations are present in the training set. For the random split, the test set consists of unseen donors.

Performance on a test set of unseen donors

Precision and recall for both outcome classes are similar for the different types of splits in training and test set. Table 5 shows the comparison in performance between the time split and the random split, as described in the methods section. Metrics are shown for SVM-5; the differences are smaller for all other models. For women, the random split has a higher precision and recall than the time split. For men, this is the other way around. For both sexes, the differences are minimal.

Feature importance and explanation of predictions

SHAP values were computed based on a random subset of 100 donations in the test set. Figure 2 shows the SHAP summary plot for the

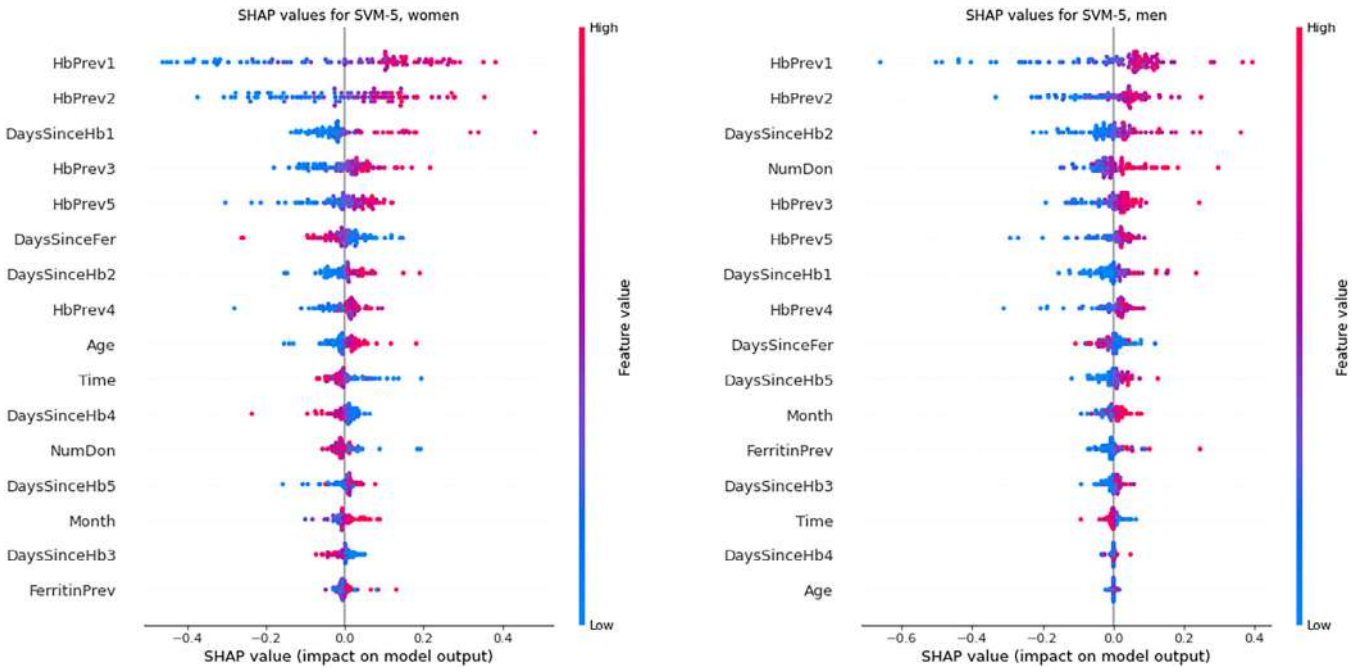

FIGURE 2 SHAP summary plots for predictions made by SVM-5, on 100 random donations from the test set. Each point represents one single observed donation. The location on the x-axis indicates the contribution of the predictor variable on the prediction (positive value: Indicative class non-deferral, negative: Indicative of class deferral) while the colour of the point indicates the relative value of the feature in that observation. The features on the y-axis are ordered by their relative importance, measured as the mean absolute SHAP value

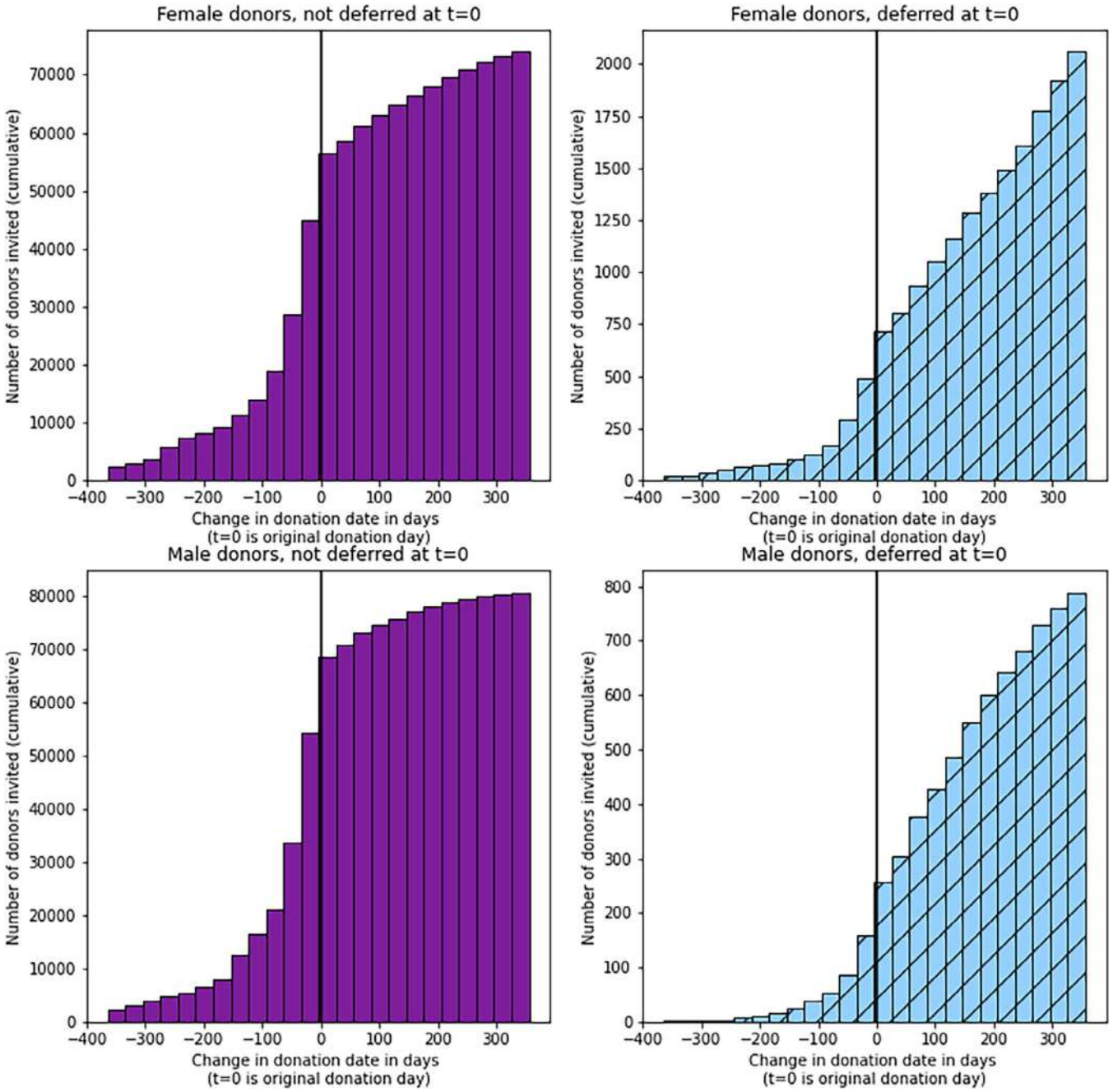

FIGURE 3 Cumulative distribution of the timing of donor invitations on basis of first predicted positive Hb level relative to the original donation date

SVM-5 models, the summary plots for the other eight models are included in Figure S1.

For all models, the most important predictor variable is the previous haemoglobin measurement (HbPrev1), and in general, more recent measurements are more important than earlier ones. The time since the previous haemoglobin measurements also ranks high on feature importance, but their chronological order is less well-preserved than the HbPrev variables.

The association between the feature value and impact on the prediction is as expected for most variables. For haemoglobin measurements, higher values are associated with predicted non-deferral. For DaysSinceHb, longer times since the previous haemoglobin measurement are indicative of predicted non-deferral. However, DaysSinceHb4 shows the opposite association, meaning that when the fourth previous measurement was long ago, the chance of predicted non-deferral becomes lower, while higher would be expected.

Variable NumDon has the expected impact on prediction in all models but SVM-5 for female donors; in all other models, a higher number of recent donations shifts the prediction towards deferral. In most models, the number of donations is a more important predictor

for men than for women, and it is always less important than all HbPrev variables.

The variable FerritinPrev shows the same association with the prediction as HbPrev variables: higher ferritin levels are associated with predicted non-deferral. Ferritin is a more important predictor for men than for women. For both sexes, the time since the previous ferritin measurement is more important than the actual ferritin level, and a higher value for DaysSinceFer makes predicted deferral more likely.

We know that for women, higher age makes deferral less likely (due to menopause), and the SHAP values confirm this relation. For men, age is one of the least important predictors, and there is no clear direction of the relation. The month of donation is of medium importance for both sexes, with predicted deferral being more likely earlier in the year. This captures the seasonal effect of temperature on haemoglobin as measured by the HemoCue. Donating earlier in the day (i.e., a lower value for variable time) increases the likelihood of predicted non-deferral, which is supported by previous research showing that haemoglobin levels are highest in the morning and decrease throughout the day [14].

Impact on blood supply

Figure 3 shows the cumulative count of donors as invited by the models relative to their original donation date. Once the model predicts non-deferral, it never predicts deferral at a later date. Of non-deferred donors, 50% would be invited more than 2 weeks earlier by the model, and 26% within 2 weeks from around the original donation date. Only 5% would not be invited within a year, causing a successful donation to be missed. Of deferred donors, only 13% would be invited earlier, while 40% would be invited over 3 months later. 28% would not be invited within 1 year. The majority of donors would be invited around their original donation date. For many donors, the original donation date was shortly after the minimum donation interval had passed, and as such, there was no room to invite them earlier.

Because the true haemoglobin level of donors on days other than their original donation date is unknown, we must make assumptions about the accuracy of the predictions in order to calculate a hypothetical number of donations and deferrals. In the most optimistic scenario, all donors who were not deferred on their original donation date would also not be deferred if they were invited earlier; and all donors who were deferred on their original donation date but are invited later by the model would not be deferred by then. In that scenario, only 5% of successful donations would be lost because the model would (incorrectly) not invite those donors, while the deferral rate would decrease by 60% (from 3% to 1% for women and from 1% to 0.4% for men).

We estimate the impact on the blood supply by comparing the length of the original donation interval to the donation interval as suggested by the model. For women (men), the median time between two donations decreases from 157 to 127 (92 to 63) days using the prediction model. Therefore, the total number of blood bank visits per time unit would increase by a maximum of 15%. This assumes that all donors who responded to the original invitation would also respond to the invitation if it would be sent at an earlier or later date. We also assume that all donors visit the blood bank within 1 week of the invitation. With the original invitations, 15% of donors that responded to the invitation visited the blood bank within 8 days, so the 15% increase in visits is likely to be a small overestimation. These assumptions may not hold for mobile donation sites but are reasonable for all regular donation sites, where 95.3% of all visits in our data occurred.

DISCUSSION

This study presents an explainable machine learning approach to predict haemoglobin deferral in whole-blood donors using the information on previous donations and various donor characteristics. We show that we can prevent up to 60% of on-site low haemoglobin deferrals using the model to guide donor invitations.

To our knowledge, this is the first model using machine learning for explainable haemoglobin deferral prediction. An explainable model outcome is crucial for prediction models that are to be used in the context of a decision-support system concerning humans. SHAP values show that our models are able to learn biologically sensible associations. They support findings from other prediction models that found the previous haemoglobin value to be the best predictor for future deferral. We add to this by showing that including more previous donations will improve these predictions.

Although most associations found by SHAP values can be explained biologically, some seem to be caused by organizational policies. Higher values for DaysSinceFer are associated with predicted deferral; the opposite association is found for DaysSinceHb variables. For donors with fewer than five donations since the start of ferritin testing, the only ferritin measurement is the one taken at their new donor intake, and therefore the time since that previous ferritin measurement is equal to the time since their new donor intake. It is known that deferral becomes more likely once a donor has been donating for a longer period of time.

The precision of class deferral is low, meaning that the predicted deferral is wrong for a substantial proportion of donors. However, by predicting deferral for different timepoints, we see a clear difference between deferred and non-deferred donors: non-deferred donors are in many cases invited earlier than their original donation date by the model, while deferred donors are mostly invited later or not at all, thereby reducing the deferral rate. In non-deferred donors, the median donation interval becomes shorter if invitations were guided by the model, and thus the number of blood bank visits per time unit would increase.

We can only calculate the accuracy of deferral predictions on the original donation date, as haemoglobin levels on other days are unknown. As haemoglobin levels slowly increase after a donation, non-deferred donors would also not be deferred if they were invited later. If they are invited earlier, we cannot know if their haemoglobin level is already above the deferral threshold. The same applies to deferred donors that are invited later by the model – it is plausible that their

haemoglobin levels are above the threshold then, but not certain. Based on accuracy measures of predictions on the original donation dates, we can be fairly confident that the predictions are reliable.

Incorporating prediction models in haemoglobin deferral policies could bring many benefits to blood banks, but it is important to think about how they should be used. If the model is used in practice, the change in policy will lead to changes in the data. Models would therefore need updating by re-training on a regular basis. Additionally, it would be wise not to outsource invitations to the model completely, as that would hinder the model's ability to learn from its mistakes. Although deferrals incorrectly predicted to be non-deferrals would be discovered, we would never know how many donors were incorrectly not invited. This can be prevented by sending part of the invitations without using the model's predictions. In addition to using the model to predict deferral outcomes, the model can also be used to return a deferral probability, allowing blood banks to incorporate this probability in their risk assessment when inviting donors.

Our model is limited to predictor variables that are presently collected by Sanquin. Additional variables could be considered to improve prediction accuracy. Donor height and weight (optionally BMI or total blood volume), as well as smoking status, are examples known to be related to iron levels and are relatively easy to be included. Information on iron-related genetic markers or donor diet may also improve accuracy but are expensive to collect.

Based on the results of this study, we conclude that using prediction models to guide donor invitations would bring multiple advantages to blood banks: lower deferral rates combined with shorter donation intervals would result in motivated and healthy donors, as well as a steady blood supply.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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SUPPORTING INFORMATION

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

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ORIGINAL ARTICLE

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Six Sigma in blood transfusion services: A dream too big in a third world country?

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Abstract

Background and Objectives: Transfusion errors can occur anywhere from blood donation to final blood transfusion. They are a source of increased cost and patient mortality. Automated workflows can reduce transcription errors, but resource-poor centres still use semi-automated/manual method for testing including manual labelling of column agglutination cards/testing tubes. Missing out any details on these cards can lead to errors in reporting results, wastage and loss of resources and effort. The aim of this study was to implement Six Sigma DMAIC (Define, Measure, Analyse, Improve and Control) methodology to reduce transcription errors while labelling gel card in immunohaematology lab to zero defect.

Materials and Methods: In this prospective study, transcription errors while manually performing 200 tests with 1400 opportunities were analysed. Baseline variables like number of errors, defects per million opportunities and sigma level in our current setup were measured. With the application of DMAIC methodology, root cause analysis for each error using Ishikawa diagram and structured Interviews were done to identify causes. A multipronged approach to deal with errors was done to improve critical areas using brainstorming sessions and developing training sheets for practice. After implementing the changes, baseline variables were reanalysed.

Results: Application of DMAIC resulted in an overall reduction in defects from 34.86% to 0.56% with sigma level improvement from 1.89 to 4.08.

Conclusion: Six Sigma methodology can be used in a resource-poor setting even with lack of automation to ensure error-free process flow.

Keywords

blood centre, quality, resource poor, Six Sigma, transfusion

Highlights

- Six Sigma methodology has been applied to reduce errors during manual testing in a resource-poor set-up.
- Blood centres in the developing world that cannot afford automation can still ensure quality in their processes by implementing Six Sigma methodology.

INTRODUCTION

Blood transfusion services is one of the most critical areas in patient care. Blood transfusion-associated mortality due to human errors has been adequately documented [1, 2]. Transfusion errors can occur anywhere along the process starting from blood donation, storage, processing, component preparation, transcription of results, accurate labelling and final blood transfusion. Despite persistent human efforts to eradicate transfusion-related errors, significant blood transfusion errors persist, and many are still underreported.

Fully automated workflow can drastically reduce the transcription errors. But there are many centres in developing world which still use semi-automated/manual method for testing. In absence of infrastructure or resources for fully automated workflow, they still have to label column agglutination cards/testing tubes manually for various serological tests like the ABO and Rh typing, patient antibody screening and crossmatch to name a few. For correctly matching the test results with the patient, details of the patient must be mentioned on the gel card. Missing out these details can lead to errors in reporting the test results, wastage of tests and ultimately loss of both resources and effort. In such setting, one often feels that certain errors are bound to happen because as the age-old adage goes 'to err is human …'

This study was planned as a project to offer and in fact develop an error-free working culture in a manual testing setup

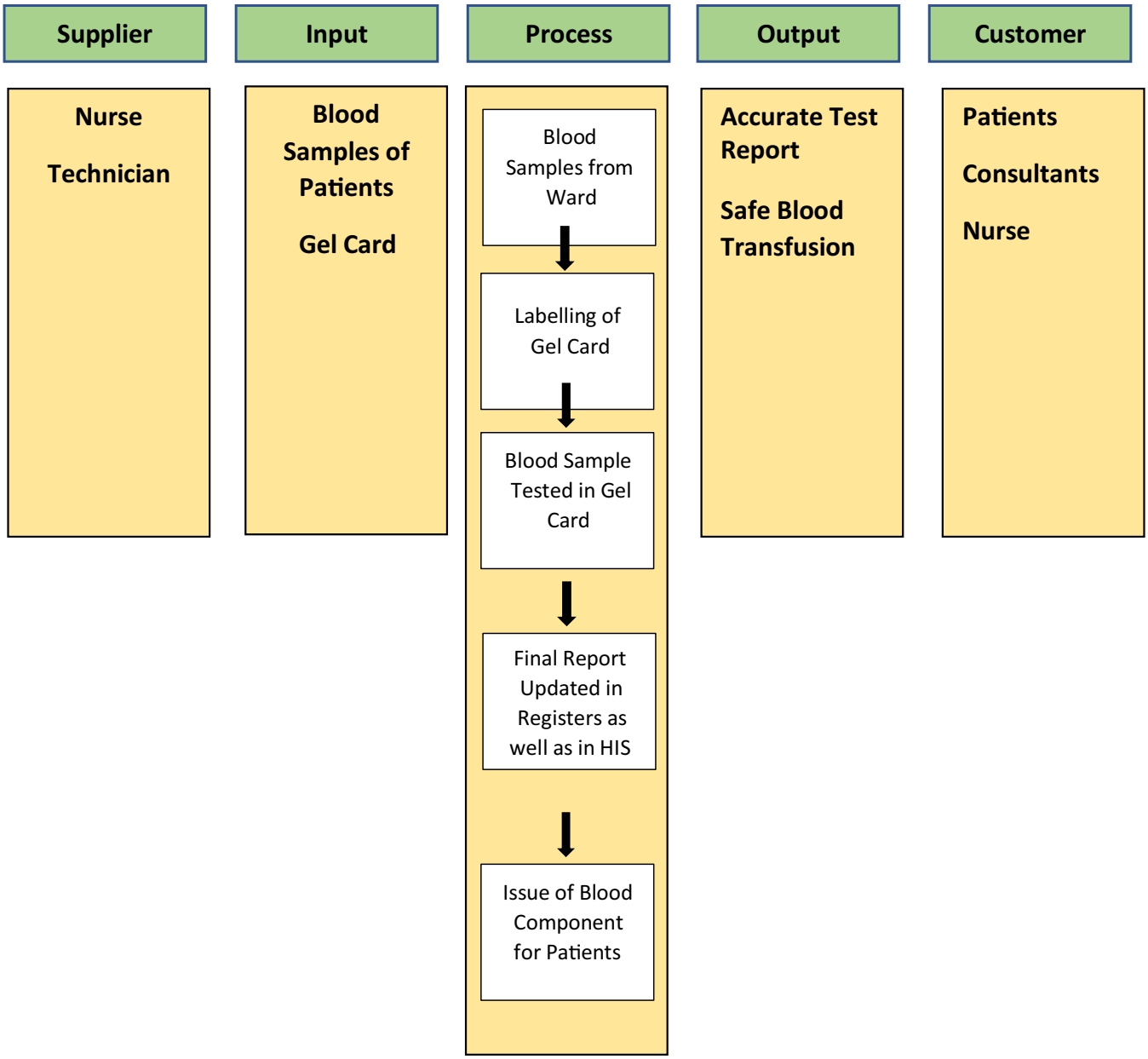

FIGURE 1 SIPOC (suppliers, inputs, process, outputs, customers) tool for visualization of testing process. HIS, Hospital Information System

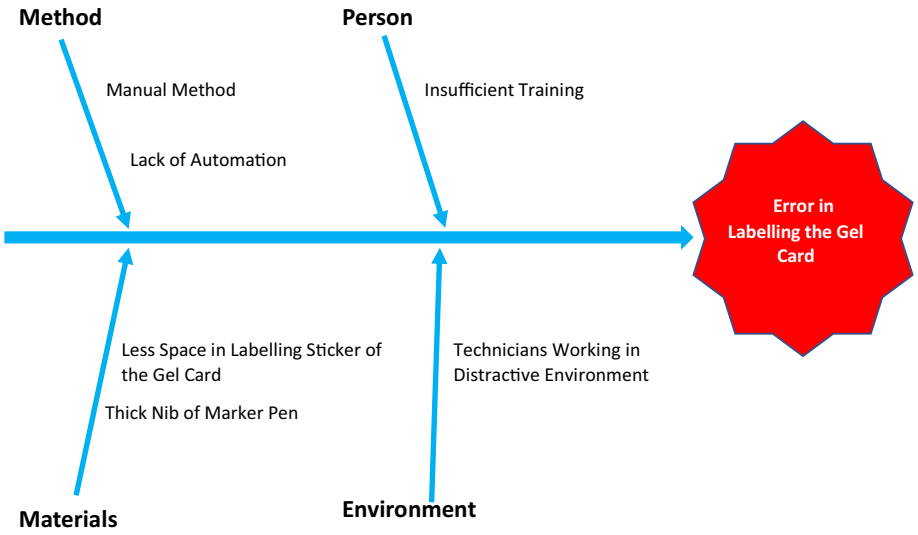

FIGURE 2 Fishbone cause–effect diagram

TABLE 1 Test-wise opportunities before and after DMAIC intervention

Name of the test	Number (before DMAIC intervention)	Number (after DMAIC intervention)
Patient antibody screening	49	45
Cross matching	87	58
ABO and Rh typing	64	97
Total gel cards	200	200
Number of opportunities	1400	1400

Abbreviation: DMAIC, Define, Measure, Analyse, Improve and Control.

TABLE 2 Number of defects before and after DMAIC intervention

	Number (before DMAIC intervention)	Number (after DMAIC intervention)
Compliance	799	1251
Non-compliance (defects)	488	7
Not applicable	113	142
Total	1400	1400
Defects %	34.86	0.50

Abbreviation: DMAIC, Define, Measure, Analyse, Improve and Control.

using Six Sigma methodology, which has withstood the test of time in improving the quality of process and reduce errors or defects. The term 'Six Sigma' was originally coined by Motorola engineer Bill Smith in 1980s [3] and has been used extensively in manufacturing industries and is increasingly finding its way into the healthcare industry to increase the quality of services and patient safety. The DMAIC (Define, Measure, Analyse, Improve and Control) methodology of Six Sigma is used to improve existing business processes and designs. Six Sigma application in blood banks so far has been limited to the improvement of quality in donor flow, blood storage and component preparation [4–6].

The aim of this study was to implement Six Sigma DMAIC methodology to reduce transcription errors while labelling gel card used during manual method of testing in the immunohaematology lab to zero defect in blood bank. To the best of our knowledge, this study is first of its kind in application of Six Sigma in transfusion services in India.

MATERIALS AND METHODS

The prospective observational study was conducted in the blood bank of a tertiary care hospital in North India over a period of 2 months. We checked 200 gel cards with 1400 opportunities and noted the errors made in those opportunities. This was followed by measures to improve the error rates and then re-evaluation of another set of 200 gel cards to see if the interventions were successful. The DMAIC methodology of Six Sigma was followed.

Define

The problem statement was identified as: transcription errors happening while labelling the gel card for seven essential parameters (name of patient, UHID i.e. Unique Hospital Identification, test name, date, time, signature and bag no. if applicable). The goal statement was to reduce the transcription errors to zero while labelling the gel card to ensure safe blood transfusion.

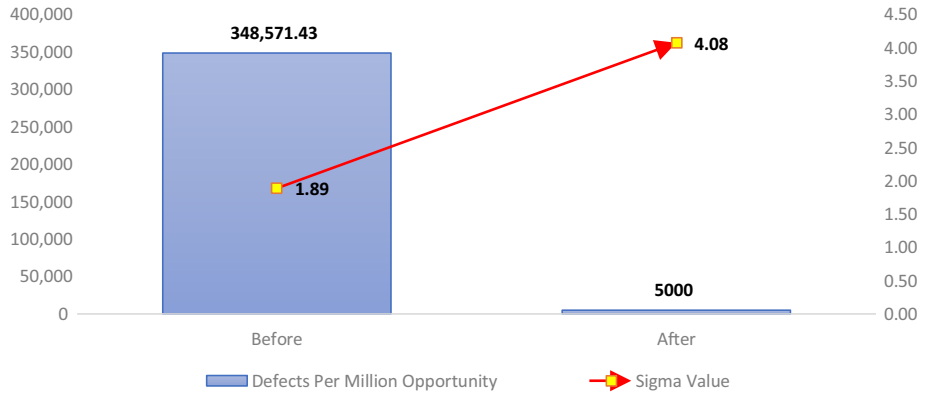

FIGURE 3 Defects per million opportunities and sigma value before and after DMAIC intervention

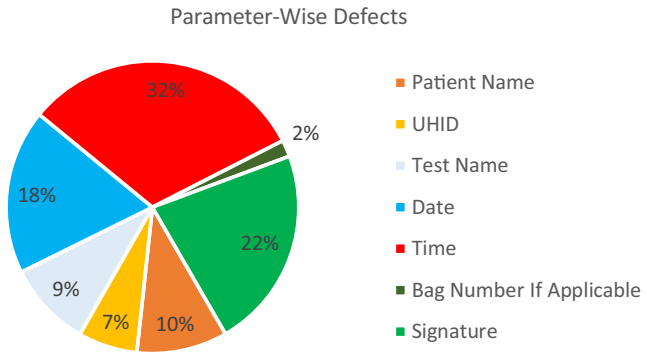

FIGURE 4 Parameter-wise defects before the intervention

TABLE 3 Defects during day shift before and after DMAIC intervention

8 AM–8 PM	Number (before DMAIC intervention)	Number (after DMAIC intervention)
Compliance	689	1028
Non-compliance	376	7
Total	1065	1035
Defects %	35.31	0.68
DPMO	353,051.64	6763.28
Sigma value	1.88	3.97

Abbreviations: DMAIC, Define, Measure, Analyse, Improve and Control; DPMO, defects per million opportunities.

Measure

The errors were measured using a checklist. The checklist consisted of the seven parameters mentioned in the define phase, which needed to be captured for the labelling in the gel card. Each parameter had the following options:

1. Compliance: when the parameter is present and legible.
2. Non-compliance (defect): when the parameter is either absent or not legible.
3. Not applicable: when the parameter was not required.

In order to ensure that there was no subjective bias in observation, we created a visual tool SIPOC (suppliers, inputs, process, outputs, customers) diagram to help understand the entire process from start to end as shown in Figure 1. Additionally, the observations were done by a non-blood bank staff to avoid observer bias.

Baseline measures were recorded. Percentage of defects was calculated as number of errors/total number of opportunities × 100. Defects per million opportunities (DPMO) were calculated as number of errors/total number of opportunities × 1,000,000. Compliance was checked with respect to the type of test and the time of the day. Sigma value was calculated in the MS Excel by the use of the following formula = NORMSINV(1 − [(total defects)/(total opportunities)]) + 1.5 [7].

TABLE 4 Defects during night shift before and after intervention

8 PM–8 AM	Number (before DMAIC intervention)	Number (after DMAIC intervention)
Compliance	110	223
Non-compliance	112	0
Total	222	223
Defects %	50.45	0.00
DPMO	504,504.50	0
Sigma value	1.49	Could not be calculated

Abbreviations: DMAIC, Define, Measure, Analyse, Improve and Control; DPMO, defects per million opportunities.

Analyse

A fishbone/cause and effect diagram (also known as Ishikawa diagram) as shown in Figure 2 was developed with the help of

brainstorming session with all the stakeholders to identify the critical causes of errors. Structured interview with the blood bank technicians was conducted to identify causes for errors. Causes such as lack of automation, less space in labelling of gel card, thick nib of marker pen, heavy workload, lack of knowledge, less manpower and a distractive environment were identified.

Improve

Measures were taken to reduce the defects based on the uncovered problems in the preceding phases. Re-audit was done post the improvement phase to check for changes in measurement if any.

During developing improvement measures, critical causes were focused on to reduce the defects.

1. Space-related issue was resolved by labelling both sides in the gel card. The gel card has both side stickers over it, but labelling was being done on only one side of the gel card. Using both the sides doubles the space available for labelling.
2. Thick nib of the marker pen with which labelling is done was replaced with a thinner nib.
3. To deal with the insufficient training, the blood bank technicians were explained the importance of doing an error-free work and to take joy and pride in immaculate work. Dummy labels were prepared matching the actual size of the gel cards and included as practice. A campaign 'Each Test is a Patient' was initiated. The technicians were randomly taken to meet the patients whose blood samples they have tested or issued blood to.
4. To deal with the issue of distracted environment, the blood bank personnel were encouraged to avoid using social media (Facebook, Twitter, Instagram, WhatsApp etc.) in the testing Laboratory

Control

Regular periodic audits were planned in this study for continuous quality improvements. The control phase of DMAIC cycle

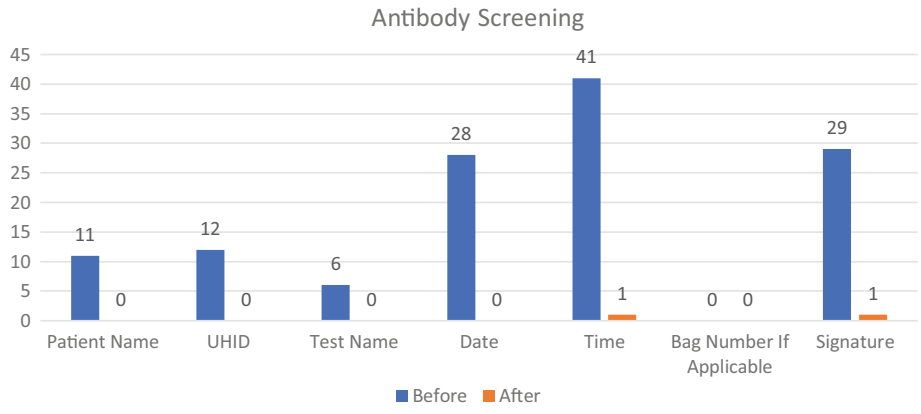

FIGURE 5 Defects seen in labelling gel cards for antibody screening before and after DMAIC (Define, Measure, Analyse, Improve and Control) intervention. UHID, Unique Hospital Identification

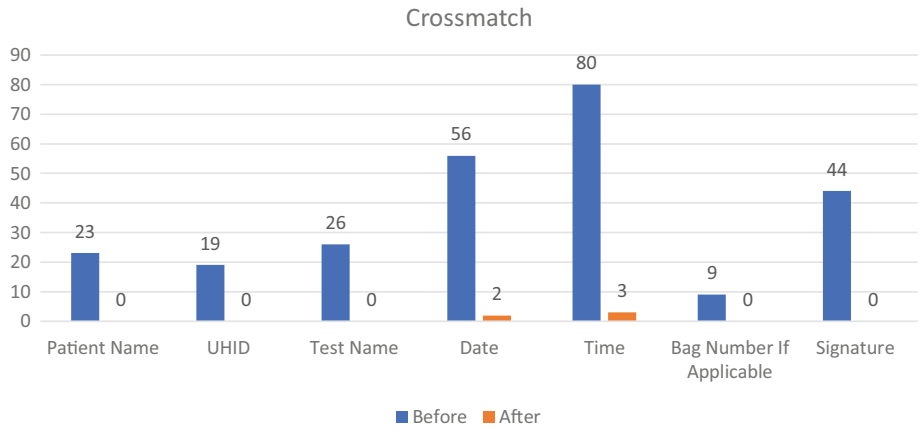

FIGURE 6 Defects seen in labelling gel cards for crossmatch before and after DMAIC (Define, Measure, Analyse, Improve and Control) intervention. UHID, Unique Hospital Identification

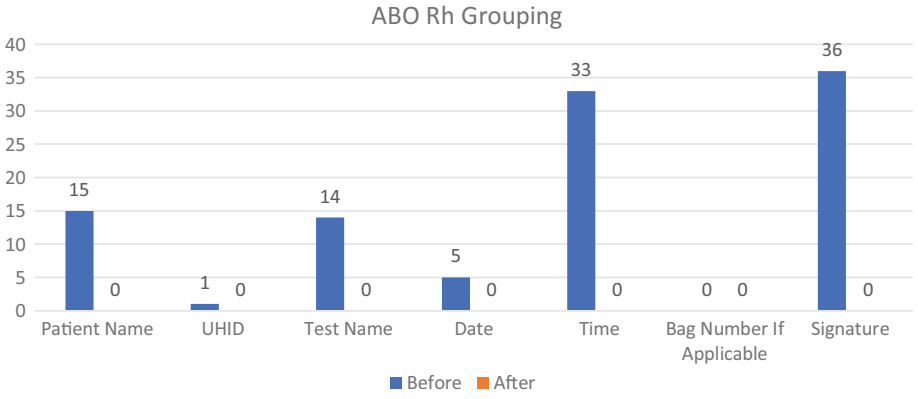

FIGURE 7 Defects seen in labelling gel cards for ABO Rh grouping before and after DMAIC (Define, Measure, Analyse, Improve and Control) intervention. UHID, Unique Hospital Identification

emphasizes on Kaizen (continuous quality improvement). Weekly review meetings and regular periodical audits were done to keep monitoring the labelling improvements.

RESULTS

In the present study, 200 gel cards with 1400 opportunities were analysed for defects both before and after the DMAIC intervention. Table 1 depicts the test-wise opportunities before and after the DMAIC intervention.

The number of parameters showing compliance was greatly increased post-DMAIC intervention from 799 to 1251, reducing the defects % from 34.86% to 0.50% which was found to be a statistically significant decrease with a p value <0.0001 (X^2 test). The number of defects seen before and after the DMAIC intervention is shown in Table 2.

Post-DMAIC intervention, the DPMO was drastically reduced from 348,571.42 to 5000 with an increase in sigma value from 1.89 to 4.08 as depicted in Figure 3.

During baseline measure, maximum number of defects were seen in case of time parameter (32%) followed by signatures (22%).

The percentage of defects corresponding to other parameters are shown in Figure 4.

To look at the incidence of defects with respect to the time of the day, the 24 h were divided into day shift (from 8 AM to 8 PM) and night shift (from 8 PM to 8 AM). We found an increased number of defects seen at night as compared to the day shift, and the increase was found to be statistically significant (p < 0.0001, 95% confidence interval, Fisher's exact test).

Post-DMAIC intervention, it was observed that the defects % decreased both during day shift from 35.31% to 0.68% and during night shift from 50.45% to 0.0%. The decrease during both day and night shift was also found to be statistically significant with a p value of <0.001 using X^2 test. The sigma values also increased during day from 1.88 to 3.97 and during night from 1.49 to 'not calculated' (due to zero error) as depicted in Tables 3 and 4.

The test-wise analysis of errors also saw significant improvement across all the test types after introduction of preventive measures. This can be seen in Figures 5–7.

DISCUSSION

In the present study, the DMAIC concept of Six Sigma was successfully implemented in the blood bank to reduce transcriptional errors while labelling gel cards and subsequently improve patient safety.

In the define phase, we created a project charter, a high-level map of the process to try and understand the needs of the customer of the process that is, the patient in this case. The problem statement and the goal of the study were identified. Transcription errors were chosen depending on the importance of the process in safe blood transfusion. Transcription errors are one of the major sources of transfusion errors [8] as cited in a 10-year study by Linden. A retrospective analysis of reports made to Serious Hazards of Transfusion shows how along with other causes, transcription errors can lead to incompatible blood transfusion and even death in certain cases [9].

In the measure phase, the team refined the definitions and determined the current performance of the baseline processes. SIPOC tool offered valuable insights into areas where major issues persisted. The plan to collect the data was formulated. A checklist consisting of seven parameters which needed to be captured for the labelling of gel cards was designed.

During baseline measurements, it was observed that out of the 1400 opportunities, 488 showed non-compliance with 34.86 defects % and a sigma value of 1.89. Majority of errors were seen while noting down the time parameter (32%). Analysis based on the time of the day showed that more defects were witnessed during the night shift as compared to the morning shift and this difference was statistically significant (p < 0.0001). Analysis based on the type of test showed more defects % in antibody screening tests (46.2%) than crossmatch (42.2%) or ABO Rh grouping (27.08%).

The analyse phase of DMAIC helped identify problems in the process that caused defects in the cycle/processes. Analyses of labelling

in the gel card for the tests showed high number of defective opportunities. The fishbone/cause–effect diagram was developed that helped identify the causes of errors. The insufficient training of the blood bank technicians arose from the fact that most of them are graduates in allied health sciences with a varied training period of 2–3 years with no formal specific training in transfusion medicine. Most of them learn transfusion medicine on the job and there is no system of obligatory Continued Medical Education (CME) in our country for the blood bank technicians. Using dummy labels helped the technicians in practising and created an open environment so that they could overcome the practical problems that were faced while labelling. The campaign 'Each Test is a Patient' had the maximum impact on the technicians. They developed a sense of responsibility and empathy towards the patient and became more conscious of their work. The impact of training in transfusion medicine has been documented by Damanhouri in his study [10]. CMEs should be conducted more frequently for the blood bank staff and should be mandatory.

With the aim to reduce the baseline defects while labelling, measures were taken, and post-implementation data collection was done with sample size of 200. It was observed that DPMO was drastically reduced from 348,571.42 to 5000 with an increase in sigma value from 1.89 to 4.08. The number of errors for each parameter was reduced to zero both during the day and night for most of the parameters.

The primary objective of the DMAIC control phase is to ensure that the gains obtained during Improve are maintained long after the project has ended. To that end, standardization and documentation of procedures were done, making sure all employees are trained and the results of the project were communicated to the staff.

Studies show that most of the Six Sigma in healthcare sector has been implemented mostly in the United States. In the Indian healthcare sector, lack of knowledge and availability of resources are the major reasons for not implementing this methodology [11, 12]. Most studies in India are empirical in nature focusing more on the management aspect rather than medical processes. Some of these studies were focused on improving hospital discharge process [13], decreasing waiting time of out-patient department (OPD) [14], improving performance and productivity in the medical records department [15], improving patient care and quality in a mobile hospital [16], and improving efficacy in ophthalmology OPD clinics using lean Six Sigma techniques [17]. In transfusion services, Divya Venugopal and others showed a reduction in donor waiting time by 50% by using DMAIC and lean to improve the management of blood donor flow [5]. Neri and colleagues used Six Sigma methodology to improve blood utilization decreasing inappropriate transfusions to less than 5% from existing 16% [18].

Six Sigma methodologies can be applied in any of the various processes involved in blood banks including (1) blood donation-creating databases of blood donors, predictive planning of blood product needs; (2) blood collection, (3) blood processing and testing, (4) stock management and (5) product distribution to improve overall management, decrease cost and increase patient safety [6].

This study has focused on the reduction of transcription error while labelling the gel cards in immunohaematology laboratory in the blood bank. We conclude that blood centres that cannot afford automation can still ensure immaculate testing practices by embracing quality concepts like Six Sigma. The benefits of addressing quality and safety problems include the obligation to improve patient care and the economic returns. At the end of the study, there is overall reduction in defects from 34.86% to 0.56% with sigma level improvement from 1.89 to 4.08.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Use of O RhD-negative red blood cells: A nationwide, prospective audit

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Abstract

Background and Objectives: Despite declining transfusion rates, overuse of O RhD-negative red blood cells (RBCs) risks the secure supply of this limited resource. A nationwide prospective audit was performed in Finland to understand the clinical use and inventory management of O RhD-negative units. Our aim was to identify areas where policy changes could help alleviate the shortage of O RhD-negative RBCs.

Materials and Methods: The use of every O RhD-negative unit in Finland during a period of 1 month was reviewed. For each issued unit ($n = 1105$), unit age, urgency of transfusion, hospital and patient demographics, and specific reasons for issuing O RhD-negative units were recorded.

Results: Almost half of the O RhD-negative units ($n = 529$, 47.9%) were issued to non-O RhD-negative patients. Only 22.3% ($n = 118$) were issued for females under the age of 50. Of the units for ABO-nonidentical transfusion, one-third (32.5%, $n = 172$) were issued for emergency transfusion, two-thirds (67.5%, $n = 357$) for non-urgent transfusions. The most common reason for issuing an O RhD-negative unit was inventory management ($n = 172$, 48.2% of units issued for non-urgent transfusion). Most of these units were issued close to the unit expiry date.

Conclusion: This nationwide audit revealed that a significant proportion of O RhD-negative RBCs are used inappropriately. Clinicians should be educated on the appropriate use of O RhD-negative RBCs, and blood banks should develop strategies for inventory management to avoid issuing O RhD-negative units purely to prevent outdating.

Keywords

emergency transfusion, inventory management, O RhD-negative, red cell components

Highlights

- Almost half of O RhD-negative red blood cell (RBC) units are issued to patients with other blood groups.
- The most common reason for issuing an O RhD-negative unit to a non-O RhD-negative patient was inventory management close to the unit expiry date.
- The inappropriate use of O RhD-negative RBCs calls for clear, uniform guidelines and urgent education of both clinicians and hospital blood banks on the appropriate use of this limited transfusion resource.

INTRODUCTION

Worldwide, the use of red blood cells (RBCs) is decreasing as clinicians increasingly adopt patient blood management principles and follow restrictive transfusion guidelines. Simultaneously, demand for O RhD-negative units has increased, and the proportion of transfused O RhD-negative units is generally up to twice as high as the proportion of the O RhD-negative blood group in the population [1–5]. Problems in recruiting enough O RhD-negative donors from an ageing donor population are universal, and the overutilization of O RhD-negative RBCs leads to supply shortages [3] and ultimately to situations where O RhD-negative RBCs are not available to patients who need them [6].

Reasons for this overuse of O RhD-negative units include emergencies, neonatal transfusions and transfusions in stem cell transplant recipients. Most blood providers still perform extended RBC antigen typing mainly to group O donors, and phenotype-matched units for alloimmunized or transfusion-dependent patients are commonly O RhD-negative. Furthermore, smaller or remote hospitals may simply lean their inventory management by storing only O RhD-negative units compatible with any patient. Therefore, one- to two-thirds of O RhD-negative RBCs are transfused to patients of other blood groups [2, 5–7].

Maintaining a sufficient inventory to secure a safe supply for O RhD-negative patients is challenging in any hospital. Too small an inventory may set O RhD-negative patients and those females of childbearing age in need of emergency transfusion at risk of RhD immunization, and too large an inventory may lead to transfusion of O RhD-negative units to patients of other blood groups to avoid wastage. Indeed, an important factor driving the use of O RhD-negative RBCs seems to be transfusion near the expiry date solely for stock management purposes [5, 7]. In their 2019 recommendation on the use of group O red blood cells, Association for the Advancement of Blood & Biotherapies (AABB) called for transfusion services to monitor and audit the utilization of group O red blood cells to limit the unnecessary use of O RhD-negative RBCs [4].

The Finnish Red Cross Blood Service (FRCBS) is responsible for supplying all blood products in Finland, a country with a population of 5.5 million, of which 5% are O RhD-negative. Since 2010, there has been a 20% decline in the use of all RBC units in Finland, while the proportion of issued O RhD-negative units has increased from 6% to over 8%, suggesting significant overuse of O RhD-negative RBCs (Figure 1). In this prospective nationwide audit, FRCBS tracked the use of O RhD-negative units in Finland over a period of 1 month in 2019. The aim was to understand the clinical use and inventory management of O RhD-negative units in Finnish hospitals and find areas where current practices could be changed to help balance O RhD-negative RBC supply and demand.

MATERIALS AND METHODS

In Finland, manufacturing of all blood products is centralized at FRCBS Helsinki Center. Half of all RBC units are stored in the three FRCBS inventories in Helsinki, Kuopio and Oulu, and the other half in hospital blood banks, which maintain stock according to the medical profiles of the hospitals. The target RBC stock level for O RhD-negative units in the FRCBS inventories is the expected four-day demand in Finland.

Data extraction

For 2019, data on all RBC units distributed to Finnish hospitals were extracted from the FRCBS blood management system (eProgesa, MAK-SYSTEM International Group).

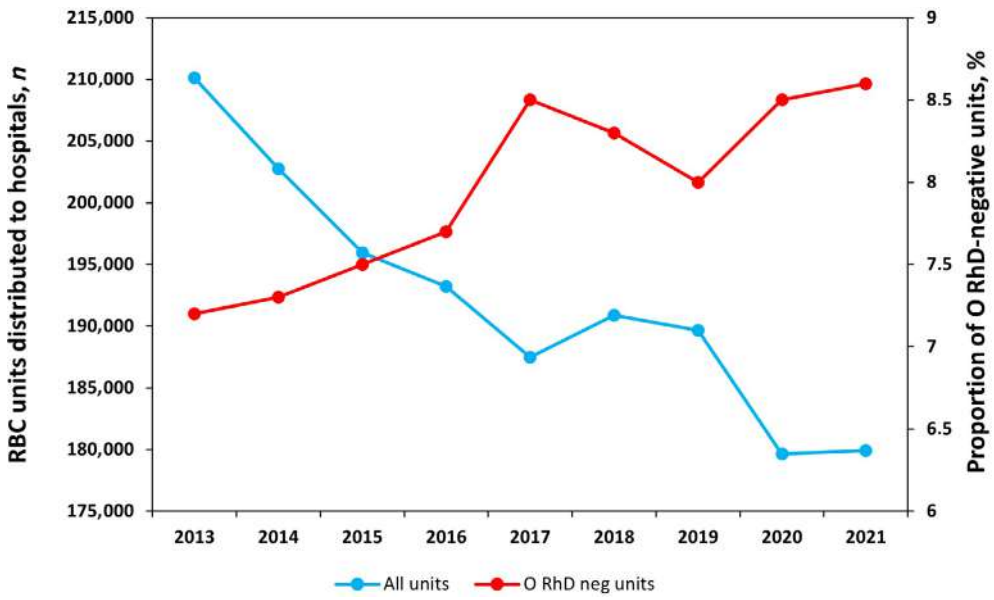

FIGURE 1 The proportion of O RhD-negative units of all distributed red blood cell (RBC) units in Finland 2013–2021

For the prospective audit, an electronic data collection form was sent to 34 blood banks in public hospitals (5 university, 17 central, 12 regional), which cover 98.9% of the RBC use in Finland. The hospital blood banks were asked to track every O RhD-negative unit issued in their service area within a 1-month period (1–30 November 2019). Both adult (250 ml) and paediatric (100 ml) size units were included in the audit. Detailed data were collected only from O RhD-negative units issued to non-O RhD-negative patients to limit the audit workload for the blood banks. For units issued to O RhD-negative patients, only the number of issued units was collected. However, the Helsinki University Hospital blood bank, serving also for one central hospital and three regional hospitals and covering a third (31% in 2019) of the O RhD-negative RBC use in Finland, collected more detailed data on O RhD-negative patients.

For the O RhD-negative units issued to non-O RhD-negative patients, the following data were collected: patient demographics (ABO and RhD group, age, sex), the reason for transfusion, the actual location of transfusion, the urgency of transfusion (emergency vs. normal), and unit age at the time of transfusion (days from expiry, DFE).

Reasons for transfusions to non-O RhD-negative patients were categorized as emergency transfusion (unknown blood group), the requirement for phenotype-matched units for alloimmunized patients, requirement for phenotype-matched units for transfusion-dependent non-immunized patients, neonatal transfusion, stem cell transplant recipients, ABO or RhD discrepancies for other reasons, inventory shortage of identical blood group, inventory management to avoid wastage of expiring units and other reason as specified by the blood bank.

Locations for transfusion were categorized as prehospital emergency medical services (EMS), hospital emergency department (ED), operating room (including delivery room), intensive care unit (ICU), neonatal intensive care unit (NICU), hospital ward for specialist care, other wards (geriatric, rehabilitation or palliative care), outpatient clinic, or home care unit.

Hospital blood bank locations were categorized according to the transfusion volumes (RBC units per year) and hospital type (university hospital, central hospital, regional hospital).

The unit age (DFE) was calculated by subtracting the unit issuing date from the the unit expiry date. In Finland, RBC units expire 35 days after collection.

For the detailed comparison between O RhD-negative and non-O RhD-negative patients, data were extracted from Helsinki University Hospital's blood bank information management system (My+ verikeskus, Mylab, Tampere, Finland). No patient identification data were handled in this extraction.

Ethics

This audit was approved by the FRCBS Research Board. As the audit was based purely on registry data from hospital blood banks, obtaining informed consent from patients was unnecessary pursuant to the Medical Research Act (488/1999) in Finland.

Statistics

Data were analysed with SPSS Version 27 (IBM Corp, Armonk, New York, USA). Data are expressed as medians (range or interquartile range, IQR) or numbers (%), as appropriate. As continuous data (patient age, unit age as DFE) distributions were skewed according to Kolmogorov–Smirnov test, the non-parametric Mann–Whitney *U* test and median test for independent samples were used for continuous variables. Categorical data were compared with the Pearson's chi-squared test or with Fisher's exact test. Correlations were assessed with Spearman rank correlation. Differences in O RhD-negative unit usage according to hospital type were assessed with Kruskal–Wallis test, and Mann–Whitney *U* test with Bonferroni correction for multiple comparisons was used for post hoc analysis. Statistical significance was set at $p < 0.05$ for all tests.

RESULTS

In 2019, the total number of RBC units distributed from FRCBS to Finnish hospitals was 189,122. Of them, 15,048 (8.0%) were O RhD-negative (Figure 1). The proportion of O RhD-negative units by hospital type and the number of all RBC transfusions are shown in Figure 2. There was a negative correlation between the proportion of O RhD-negative units issued and the total number of RBC units distributed to the hospital. For the university hospitals, which accounted for 53.3% of all distributed RBC units in 2019, the median proportion of O RhD-negative units was 7.0% (range 6.3%–7.7%). For the central hospitals and regional hospitals, the corresponding proportions were 8.1% (range 5.3%–15.5%) and 13.9% (range 8.1%–34.7%), respectively. The variation between hospital types was significant ($p < 0.001$, Kruskal–Wallis test) (Figure 2).

The target stock level for O RhD-negative units in FRCBS inventories (expected four-day demand) was not reached for 98 days (26.8% of the time) during 2019.

Audit response rate

All 34 hospital blood banks in Finland participated in the audit. During November 2019, a total of 1105 O RhD-negative units were issued to patients from the blood banks; the median number per blood bank was 21 (range 0–391) units. Four blood banks (4/34, 11.8%) in local hospitals did not issue any O RhD-negative units during the audit period. With the total number of O RhD-negative units distributed to hospital blood banks during 2019 taken into account, we estimated that this audit covered almost 90% of the actual use.

The reported discard rate of O RhD-negative units was 1.4% (16 units). These units were issued from the hospital blood banks but not transfused at the hospital ward and had to be discarded after remaining out of cold storage for over 1 h. None of the blood banks reported discarding any O RhD-negative units due to outdating. However, as this audit focused on units issued from the blood bank, we could not ascertain that all issued units were actually transfused.

Patient blood groups and demographics

Of the 1105 O RhD-negative units, 576 (52.1%) were issued to O RhD-negative patients, 472 (42.7%) to patients with another blood group, and 57 (5.2%) to patients with an undetermined blood group. Almost a third (32.1%) of O RhD-negative units were issued for ABO-nonidentical (A/B/AB) transfusion. The distribution of issued O RhD-negative RBC units by patient blood group is shown in Table 1. Altogether, 529 (47.9%) O RhD-negative units were issued to non-O RhD-negative patients; 40.8% of these units (216/529 units) were issued to patients younger than 50 years, and 22.3% (118/529 units) were issued to females of childbearing potential (defined as ≤50 years old). The distribution of O RhD-negative units issued to non-O RhD-negative patients by patient age and sex is shown in Figure 3.

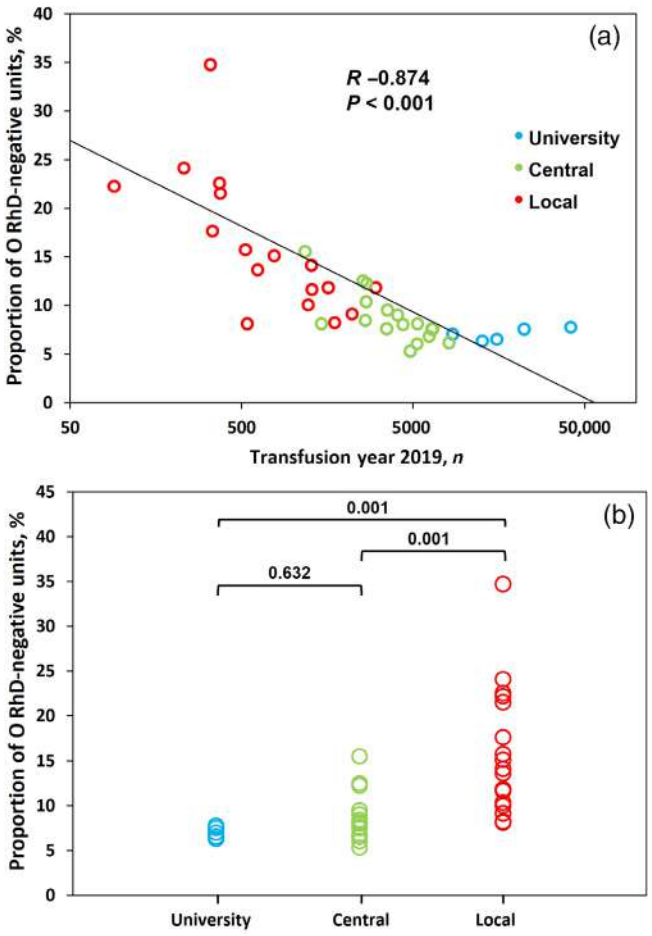

FIGURE 2 Hospital characteristics and O RhD-negative transfusions. (a) The use of O RhD-negative units vs. the number of all red blood cell transfusions in each hospital in 2019; (b) The use of O RhD-negative units according to hospital type. *p*-values are for Mann–Whitney *U* test with Bonferroni correction

TABLE 1 The distribution of transfused O RhD-negative red blood cell units by patient blood group in Finland

Patient blood group	O RhD-negative units issued in Nov 2019, % (*n*) *n* = 1105	Distribution of blood groups in the Finnish population, %	Distribution of blood groups in Finnish blood donors, %
O RhD pos	15.7 (174)	28	29
O RhD neg	52.1 (576)	5	8
A RhD pos	11.9 (131)	35	35
A RhD neg	7.1 (78)	6	7
B RhD pos	3.6 (40)	16	12
B RhD neg	2.3 (25)	2	3
AB RhD pos	1.4 (16)	7	5
AB RhD neg	0.7 (8)	1	1
Undetermined	5.2 (57)	NA	NA

Emergency transfusions to non-O RhD-negative patients

Of the 34 blood banks, only 17 (50.0%) reported at least one emergency transfusion during the audit. Of the 529 units issued to non-O RhD-negative patients, 32.5% (172/529 units) were issued for emergency transfusion: 57.0% (98/172 units) to patients over 50 years of age, 65.1% (112/172 units) to males, and only 20.3% (35/172 units) to females of childbearing age (defined as ≤50 years old). Of all the O RhD-negative units issued, 12.4% could have been spared if only females ≤50 years old were given O RhD-negative RBCs for emergency transfusion (137/1105 units were issued to males or females >50 years old).

Most emergency transfusions took place in the prehospital EMS (32/172 units, 18.6%) or the ED (69/172, 40.1%), followed by the operating or delivery room (26/172, 15.1%) and ICU (25/172, 14.5%). The rest (17/172, 9.9%) were transfused in other inpatient settings. Data on the location of emergency transfusion were not available for 5 units (2.9%).

Of the O RhD-negative units reported as discarded, 10 out of 16 (62.5%) were issued for emergency transfusion.

Non-urgent transfusions to non-O RhD-negative patients

Of the 529 units issued to non-O RhD-negative patients, 357 (67.5%) were issued for non-urgent transfusions (Figure 4). The main reason was inventory management, with 172 (32.5% of all units issued to non-O RhD-negative patients; 48.2% of units issued for non-urgent transfusion) O RhD-negative units issued purely to prevent outdating. The second most common reason was the requirement for phenotype-matched blood (11.5%, 41/357 units). Other reasons (Figure 4), as specified by the blood bank, were miscellaneous, including temporary malfunction of the blood bank information management system (30.6%, 11/36 units) and undocumented/unknown reasons (22.2%, 8/36 units).

Almost half (41.8%, 146/349 units) of the O RhD-negative units were issued to hospital wards (Figure 5). Data on the location of transfusion were not available for 11 (3.1%) units.

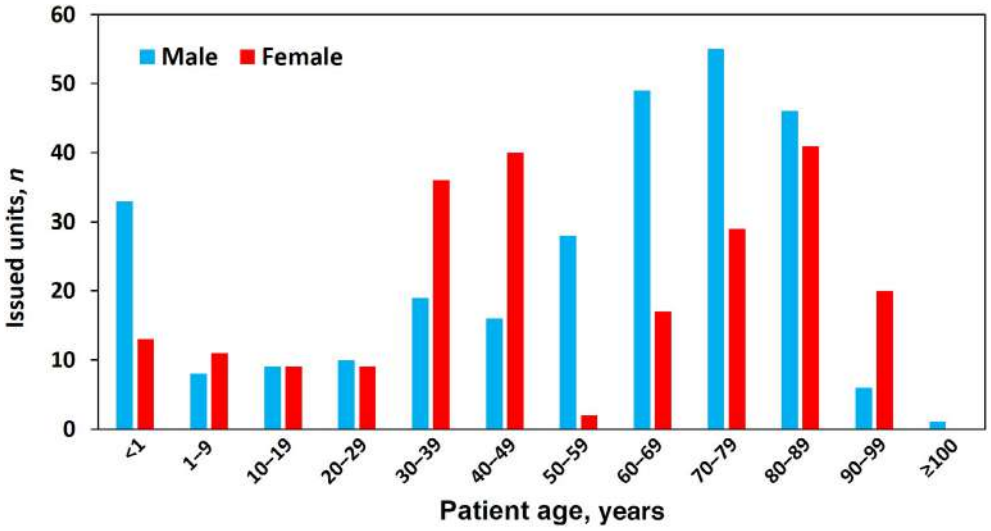

FIGURE 3 Age distribution for non-O RhD-negative patients receiving O RhD-negative red blood cells (RBCs). Data are for each issued unit; individual patients may have received more than one unit of O RhD-negative RBCs

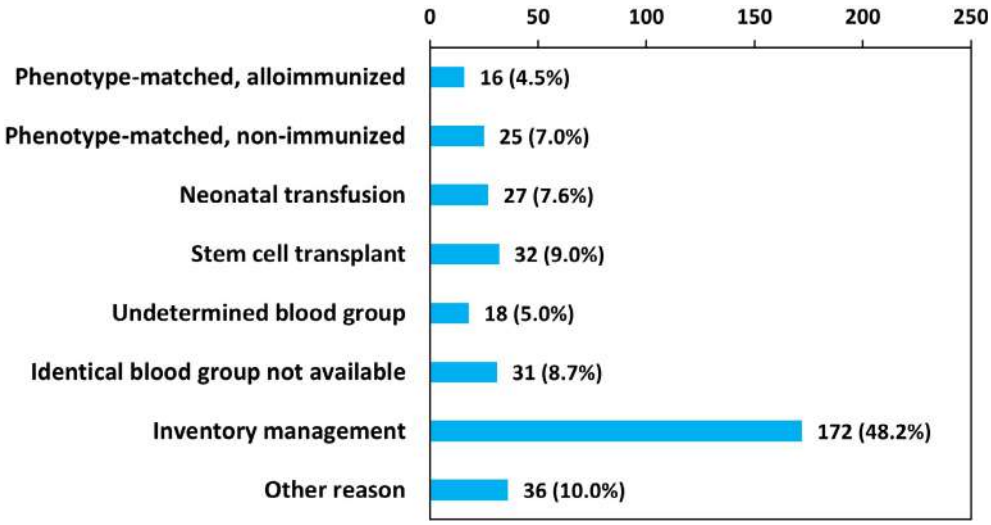

FIGURE 4 Reasons for non-urgent O RhD-negative unit transfusions to non-O RhD-negative patients. Data are as *n* (%)

Unit age at the time of transfusion

During the audit period, the median age of all issued O RhD-negative units was 24 (1–35) days, that is, median age 11 (0–34) days before unit expiry (DFE).

The median age of issued units was 22 (1–35) days in O RhD-negative and 26 (3–35) days in non-O RhD-negative patients. Units issued to O RhD-negative patients had significantly longer time to expiry [DFE 13 (0–34) days] than units issued to non-O RhD-negative patients [DFE 9 (0–32) days; $p < 0.001$ Mann–Whitney *U* test, and $p = 0.001$ median test for independent samples]. The unit age distribution as DFE is shown in Figure 6.

Altogether 82 O RhD-negative units were issued at expiry date (DFE ≤1 day); 17 units were issued to O RhD-negative patients, and 65 units to non-O RhD-negative patients ($p < 0.001$, Pearson's chi-squared test). Similarly, a higher proportion of O RhD-negative units at DFE 5 days and DFE 10 days were issued to non-O RhD-negative patients (data not shown; $p < 0.001$ for both DFE 5 days and DFE 10 days, Pearson's chi-squared test), indicating that O RhD-negative units close to expiry were issued primarily to non-O RhD-negative patients.

Comparison between O RhD-negative and non-O RhD-negative patients

For comparing O RhD-negative and non-O RhD-negative patients in detail, complete data on patients were extracted from Helsinki University Hospital's blood bank information management system, accounting for 35.4% (391/1105) of all O RhD-negative units issued in Finland during the audit. In Helsinki University Hospital blood bank,

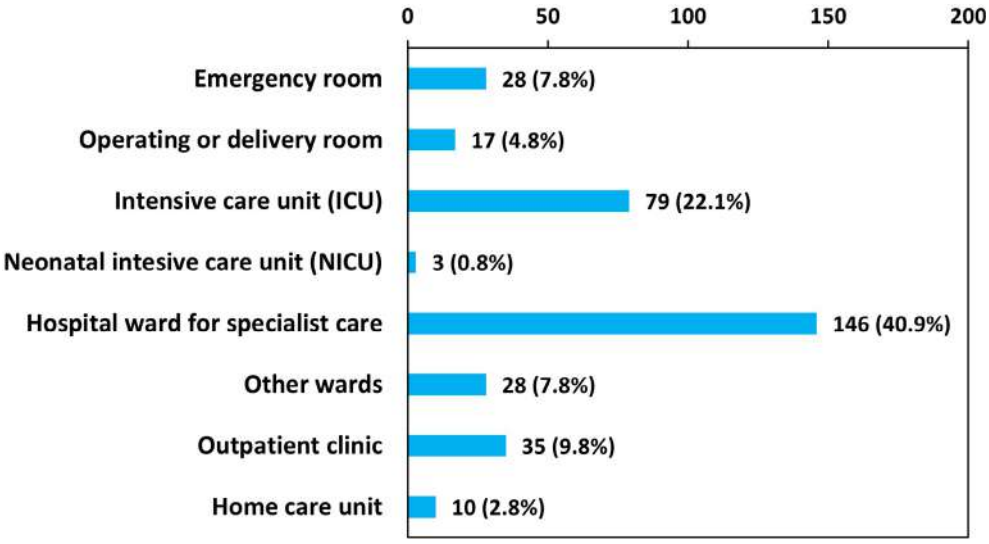

FIGURE 5 Locations of non-urgent O RhD-negative unit transfusions to non-O RhD-negative patients. Data are as *n* (%). The location of transfusion was unknown for 11 (3.1%) issued units

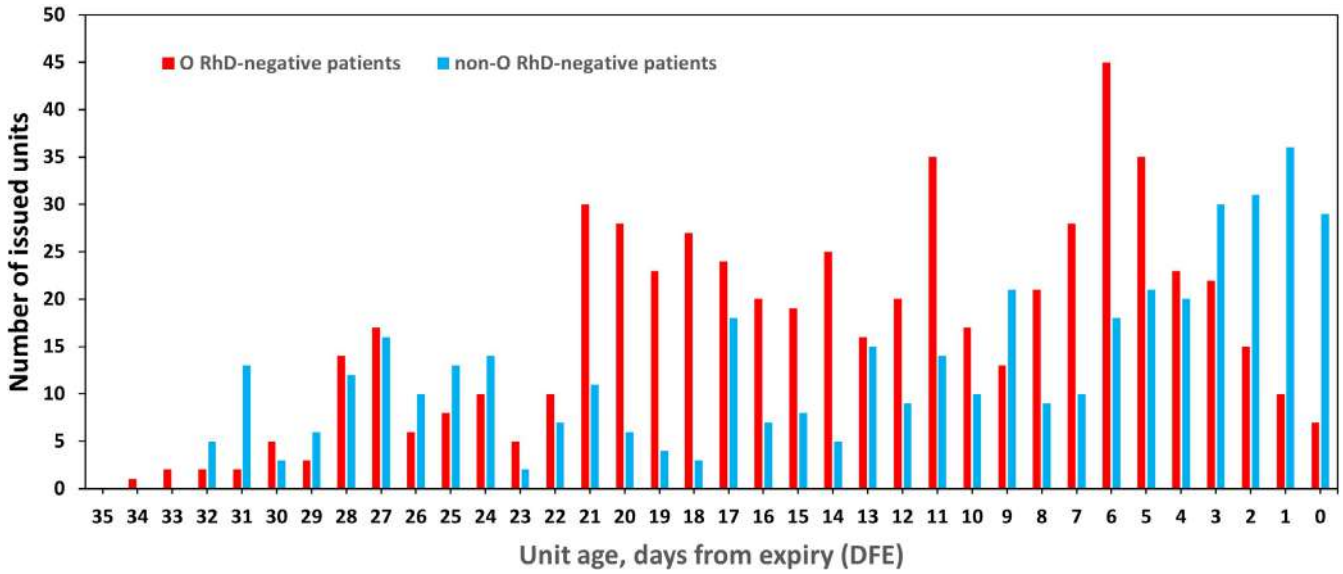

FIGURE 6 Unit age (days from expiry) at the time of transfusion

216 units (55.2%) were issued to O RhD-negative patients and 175 units (44.8%) to patients with another blood group, including those with undetermined blood group.

O RhD-negative patients were significantly older [68 (0–94) years] than non-O RhD-negative patients [39 (0–100) years; $p < 0.001$, Mann–Whitney *U* test]. In O RhD-negative patients, the sex distribution was equal (male $n = 111$, 51.4%; female $n = 105$, 48.6%), whereas in non-O RhD-negative patients, O RhD-negative units were issued more often to male ($n = 115$, 65.7%) than female patients ($n = 60$, 34.3%, $p = 0.004$, Pearson's chi-squared test). This is partly explained by the slightly, yet not statistically significant, higher proportion of emergency transfusions in male patients (male 42/115 units; female 14/60 units; $p = 0.076$, Pearson's chi-square test).

Although the median number of units issued per patient was similar [O RhD-negative 2 (1–14) vs. other blood groups 2 (1–20); $p = 0.253$, median test for independent samples], this was due to a few outlier patients in both groups receiving over 10 O RhD-negative units. After omitting these patients, O RhD-negative patients understandably received more O RhD-negative units per patient (IQR 2–3 units) than patients in other blood groups (IQR 1–2 units, $p = 0.008$, Mann–Whitney *U* test).

DISCUSSION

The overall RBC use in western countries continues to decline. For various reasons, the use of O RhD-negative RBCs has not decreased

accordingly, and the demand for O RhD-negative RBCs relative to the number of O RhD-negative donors is becoming unsustainably high [1–5]. In Finland, the proportion of transfused O RhD-negative units was twice the proportion of this blood type in the donor population in the mid-2010s. A nationwide prospective audit was conducted in 2019 to determine the reasons for this alarming trend. Although performed just before the COVID-19 pandemic, we find the result highly relevant, as during the pandemic, the proportion grew even higher.

In this audit, 48% of O RhD-negative RBCs were used for non-O RhD-negative patients. This is in line with previous studies reporting similar (35%–45%) proportions [2, 6, 7]. By far, the most common reason for issuing an O RhD-negative unit to a non-O RhD-negative patient was inventory management: A third (33%) of all O RhD-negative units issued to non-O RhD-negative patients were issued purely to prevent outdating, mostly for non-urgent care in regular hospital wards. Other studies have also identified inventory management as a frequent cause of unnecessary use of O RhD-negative RBCs [2, 3, 5, 7]. Understandably, to ensure patient safety in a country where geographical distances are a major issue, many remote blood banks wish to stock enough O RhD-negative units. However, having readily available O RhD-negative RBCs also increases their use [8]. Thus, excess stock relative to the actual need ends up being the main driver of O RhD-negative RBC use at the hospital blood bank level, and the shortage of O RhD-negative RBCs at the national blood service level is therefore largely self-inflicted. Our results call for a critical evaluation of current stocking levels of O RhD-negative RBCs in hospital blood banks. Virk and colleagues [5] reported that reducing the daily stocking level of O RhD-negative RBCs by 10% led to units being transfused earlier in their shelf life and the overall rate of inappropriate O RhD-negative transfusions being reduced, without creating shortages of O RhD-negative RBCs.

Another problematic practice in inventory management we identified was the overreliance on O RhD-negative RBCs as a "universal" packed red cell product. O RhD-negative units accounted for up to 35% of all transfusions in small local hospitals, yet only half of the blood banks (all hospital types included) reported any emergency transfusions during the audit. Furthermore, close to 10% of O RhD-negative RBCs for non-urgent transfusion were issued because type-specific RBCs were not available. These practices may slightly reduce the total RBC wastage by preventing outdating of especially B and AB units, but also unnecessarily increase demand for O RhD-negative RBCs. Despite logistic challenges, the most obvious solution is to increase the number of available B and AB units in smaller local hospitals and then circulate units a few weeks before the expiry date to a bigger tertiary hospital in the same geographical area, a policy already commonly used in several countries for O RhD-negative units [1–3, 8]. Beckman and colleagues [1] have also proposed that the overuse of O RhD-negative RBCs in smaller hospitals may reflect insufficient transfusion medicine expertise in the local blood bank, a problem solved only with continuous education.

Guidelines recommend that O RhD-negative RBCs, when not intended for group-specific transfusion, should be used primarily for females of childbearing potential who are either known to be RhD-negative or for emergency transfusion before the completion of pretransfusion testing [4, 9]. In this audit, only a fifth (22%) of all O RhD-negative units were issued to this patient group (defined as any female ≤50 years old). For emergency transfusions, a similar rate (20%) was observed. If O RhD-positive RBCs were used for emergency transfusions for men and postmenopausal women, as recommended [1, 4], 12% of O RhD-negative units issued during this audit could have been saved. Switching to O RhD-positive RBCs in all emergency transfusions has yielded similar (10%–12%) reductions in O RhD-negative RBC use [10, 11]. The tradition of using only O RhD-negative RBCs for emergency transfusion is strong in Finland. Thus, national guidelines for using O RhD-positive RBCs for emergency transfusion for males and postmenopausal females are urgently needed. The guidelines should also clearly define which patient groups can be switched to O RhD-positive RBCs for non-urgent transfusions at times of O RhD-negative RBC stock shortages [1, 4, 9]. These policy changes require thorough education in the hospital blood banks, which are responsible for selecting the units for issuance, and provision of information to the clinical stakeholders.

We also identified other worrisome practices in emergency transfusions. Although most emergency transfusions took place in the prehospital EMS or the ED, a third took place in the operating room and ICU. This suggests inadequate preparation for transfusion, as pretransfusion testing should have been possible for most of these patients in advance, enabling ABO-identical transfusion. Another practice raising concern was that a majority of discarded O RhD-negative units were initially issued for emergency transfusion. The units were issued from the blood bank, but unused units could not be stock after remaining out of cold storage for too long. This demonstrates that the threshold for clinicians ordering RBCs for emergency transfusion is too low, and more importantly, the personnel in the EDs are not familiar with the correct storage of RBCs. Both problems could be mitigated by education, and wastage might also be reduced by stocking a small amount of group O (RhD-positive) RBCs in the ED [10, 12].

Group O RBCs are commonly used for neonatal transfusion, stem cell transplant recipients and when extended phenotyped RBCs are required [1, 4]. In this audit, 12% of the O RhD-negative units were issued due to the need for extended phenotyped RBCs. To reduce this burden on the O RhD-negative RBC stock, FRCBS has, since the audit, started to expand extended phenotyping to group B donors, along with O and A donors. As for neonatal transfusion, blood banks serving hospitals with neonatal intensive care units should be encouraged to set up infant testing for maternal ABO-immunization, which would allow the use of electronic crossmatch for ABO-identical RBCs.

The widespread overuse of O RhD-negative RBCs observed in this study is problematic not only because of periodic shortages but also for clinical reasons. A considerable proportion of the O RhD-negative units were issued for ABO-nonidentical transfusion. ABO-compatible nonidentical transfusions are associated with increased patient morbidity and mortality [13, 14]. The results of our study suggest that blood bank personnel, who select RBC units for issuance, should be educated on the fact that ABO-identical transfusions are most likely the safest practice from a clinical perspective, and ABO-nonidentical transfusions should be avoided when possible.

The strength of this study is its prospective and nationwide nature. All 34 hospital blood banks in Finland participated in the audit. The results thus give a reliable view of current practices in O RhD-negative RBC use in a developed country with comprehensive public health care and a highly uniform clinical transfusion policy. The audit, to gain a realistic view of everyday practices in hospital blood banks and wards, was performed in winter instead of summer when elective surgery is scaled down for holidays, reducing the use of RBCs. Although conducted just before the COVID-19 pandemic, the audit is topical as the share of O RhD-negative units continued to increase throughout the pandemic. However, there are a few limitations to this study. First, as the primary focus of this audit was to describe the use of O RhD-negative RBCs in ABO-nonidentical transfusions, that is, to patients from blood groups other than O RhD-negative, the comparison to patterns of RBC use in O RhD-negative patients is inevitably limited. However, data on O RhD-negative transfusions to O RhD-negative patients were available from Finland's biggest hospital blood bank, Helsinki University Hospital blood bank, accounting for approximately a third of RBC use in Finland. Second, we could not ascertain whether all issued units were actually transfused. Although hospital blood banks operate on electronic blood bank information management systems and most hospital units and wards record transfusions to electronic health records, some EDs and primary care units still rely on physical health records for documenting transfusions. We did not have access to these records. For example, in the Helsinki University Hospital blood bank, 17% of issued units could not be confirmed as transfused.

In conclusion, almost half of the O RhD-negative units are issued to patients with other blood groups, and the most common reason for this practice is inventory management. With every third O RhD-negative unit issued simply to prevent outdating, clearly, the hospital blood bank O RhD-negative RBC stocks are excessive in relation to the actual need. Furthermore, we identified several harmful practices related to emergency transfusions, particularly using only O RhD-negative RBCs in in-hospital settings. Our results call for a critical evaluation of blood bank inventory management practices and education for the key stakeholders on the appropriate use of O RhD-negative RBCs. Most importantly, clinicians need to understand the difference between the ABO and RhD groups in blood transfusions. These actions could help ensure a sustainable supply of O RhD-negative RBCs in the future.

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S.S. planned the study; all authors participated in the collection of the data; M.I. performed statistical analysis; M.I. wrote the first draft of the manuscript; S.S. and K.S. reviewed and edited the manuscript; all authors accepted the final version of the manuscript.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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ORIGINAL ARTICLE

Vox Sanguinis ISBT International Society of Blood Transfusion

Modelling the outcomes of different red blood cell transfusion strategies for the treatment of traumatic haemorrhage in the prehospital setting in the United Kingdom

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Abstract

Background and Objectives: The limited supply and increasing demand of group O RhD-negative red blood cells (RBCs) have resulted in other transfusion strategies being explored by blood services that carry potential risks but may still provide an overall benefit to patients. Our aim was to analyse the potential economic benefits of prehospital transfusion (PHT) against no PHT.

Materials and Methods: The impact of three PHT strategies (RhD-negative RBC, RhD-positive RBC and no transfusion) on quality-adjusted-life-years (QALYs) of all United Kingdom trauma patients in a given year and the subset of patients considered most at risk (RhD-negative females <50 years old), was modelled.

Results: For the entire cohort and the subset of patients, transfusing RhD-negative RBCs generated the most QALYs (141,899 and 2977, respectively), followed by the RhD-positive RBCs (141,879.8 and 2958.8 respectively), and no prehospital RBCs (119,285 and 2503 respectively). The QALY difference between RhD-negative and RhD-positive policies was smaller (19.2, both cohorts) than RhD-positive and no RBCs policies in QALYs term (22,600 all cohort, 470 for a subset), indicating that harms from transfusing RhD-positive RBCs are lower than harms associated with no RBC transfusion. A survival increase from PHT of 0.02% (entire cohort) and 0.7% (subset cohort) would still make the RhD-positive strategy better in QALYs terms than no PHT.

Conclusion: While the use of RhD-positive RBCs carries risks, the benefits measured in QALYs are higher than if no PHT are administered, even for women of childbearing potential. Group O RhD-positive RBCs could be considered when there is a national shortage of RhD-negative RBCs.

Keywords

blood transfusion, prehospital setting, quality-adjusted life years, trauma

Barnaby Roberts and Laura Green are joint first authors.

Highlights

- The difference in quality-adjusted life-years (QALYs) between group O RhD-negative and O RhD-positive red cell transfusion was much smaller than that between O RhD-positive and no prehospital transfusion (PHT).
- The benefits of transfusing group O RhD-positive red cells, measured in terms of QALYs, are substantially greater than if no prehospital transfusion is administered, even for RhD-negative women of childbearing potential.

INTRODUCTION

Several observational studies have demonstrated survival benefits following the administration of red blood cells (RBCs) or low titre group O whole blood (LTOWB, herein referred to as RBCs) compared to saline alone in the resuscitation of traumatic haemorrhagic patients in the prehospital environment [1–3]. Randomized controlled trials (RCTs) that have compared prehospital blood transfusion to crystalloids have shown different outcomes due to differences in the patients' underlying demographics and clinical environments in which the studies were conducted. An RCT in which the standard of prehospital care was supplemented with two units of plasma for trauma patients transported to the hospital by helicopter showed that plasma transfusion significantly improved 30-day survival by approximately 10% [4], with the secondary analysis showing that the combination of RBC and plasma produced the highest survival rates compared to not providing any blood products while *en route* to the hospital [5]. A recent RCT (RePHILL trial) showed that RBC transfusion plus lyophilized plasma in the prehospital setting was not superior to saline resuscitation for improving tissue perfusion or reducing episode mortality; however, at 24 h, the adjusted average differences in mortality were 7% lower in the blood components arm (personal communication from trial investigators) [6].

In the prehospital setting, group O RhD-negative RBC is transfused to avoid harms caused by the RhD-positive blood being transfused to RhD-negative recipients because the recipient's ABO/RhD type is known. These harms include severe haemolysis and possibly death [7, 8], a haemolytic transfusion reaction (HTR) following the transfusion of an RhD-positive RBC unit to a recipient with preformed anti-D [9, 10], and for the RhD-negative female of childbearing potential (FCPs) who has become D-alloimmunized through RhD-positive blood, this antibody can cause haemolytic disease of the foetus and newborn (HDFN) should she later become pregnant.

However, the ability to provide RhD-negative RBCs for prehospital transfusion (PHT) is constrained by donor supply. The current demand for group O RhD-negative RBCs in England is 13% of the supply compared to the 7% frequency of group O RhD-negative in the general population. An international study of blood centre collectors found that only 10% of their RBC distributions to hospitals were group O-negative [11]. Therefore, the supply of these precious products is very limited. Several studies have modelled the overall clinical risk of foetal/neonatal outcomes following the transfusion of RhD-positive RBCs to injured RhD-negative recipients and FCPs [12–15]. However, evaluation of the risk–benefit ratio of providing group O RhD-positive RBCs to trauma patients in terms of quality-adjusted-life-years (QALYs) gained has not been evaluated before.

In this study, we compare the relative advantages of different policies for providing PHT, whereby the effect on the recipient's QALY was modelled in the following three scenarios: using RhD-negative RBCs for all PHTs, using RhD-positive RBCs for all PHTs, or not administering PHTs to injured patients.

METHODS

We modelled the impact of three scenarios on two groups of trauma patients: (i) a representative sample of all United Kingdom trauma patients in a given year, and (ii) the subset of patients who were RhD-negative females of childbearing potential <50 years old (referred as FCP), by simulating the harms experienced by patients over the remainder of their lifetimes. In the case of HDFN occurrence, we also modelled its impact on the affected babies.

A cohort was defined as a representative sample of patients who suffered a trauma in the United Kingdom in 1 year (5561 patients), as calculated previously [16]. For post-trauma life expectancy, we used the Office for National Statistics data and set this to 48 years after the accident for the entire cohort and 56 years after the accident for the subset cohort of FCPs due to their lower age at the time of injury, accounting for the average age of trauma patient and the impact of the trauma on life expectancy [17].

We modelled the reductions in health-related quality of life (HRQoL) following the trauma using a health economics approach to capture the QALYs associated with each transfusion scenario. To calculate QALYs associated with each transfusion scenario, we obtained the HRQoL associated with each post-trauma outcome and modelled the number of years patients would spend in different health states to attain the QALYs experienced. HRQoL evidence was obtained by conducting a systematic literature review to find studies using EQ-5D [18], SF-36 [19] and HUI-3 [20] stated preference questionnaires.

Modelling impact on patients suffering trauma

For each three resuscitation scenarios (prehospital RhD-positive RBC transfusion, or RhD-negative RBC transfusion, or no PHT) and the

two patient groups (overall cohort and FCPs), we 'allocated' patients to six health states in each year following transfusion (Figure 1):

1. Patient survives trauma without harm
2. Patient dies following trauma
3. Patient survives trauma but experiences HTR morbidity
4. Patient survives trauma but dies from HTR
5. Patient survives trauma but has a child with severe HDFN morbidity
6. Patient survives trauma but has a child with HDFN mortality

Allocations 3–6 were only relevant when RhD-positive blood was transfused, and the recipient was RhD-negative. The probability of trauma survival and death was based on values obtained from two studies [1, 2] demonstrating survival benefits following PHT of injured patients. The probability of suffering transfusion harms was based on a previously published model [15]. Key inputs used for the model are provided in Table A1 in Supporting information. The RePHILL trial performed in England showed that prehospital RBC transfusion plus LyoPlas was not superior to saline resuscitation in reducing the composite outcome of episode mortality and lactate clearance in trauma bleeding patients [6]. However, the study's design and findings have been criticized, including the selection of the mortality time point in the primary outcome, the length of time elapsed from injury until the administration of the study intervention, the highly injured nature of the patients and their correspondingly high death rate [21, 22]. There was, however, a 7% absolute risk reduction (25% relative risk reduction) among the PHT recipients at 3 h compared to those who were resuscitated with saline. Thus, the sensitivity analysis was designed to include this risk reduction.

Where a patient was predicted to have a future child with severe HDFN morbidity due to RhD-positive RBCs during the resuscitation, we modelled a QALY impact to both the patient and child, where the impact to the patient is based on the impact to HRQoL observed for mothers of children with Cerebral Palsy, as there were no other published data on the impact of HRQoL for mothers of children with HDFN [23–25]. For patients that experience foetal death due to HDFN caused by anti-D that was formed following receipt of RhD-positive RBCs during trauma resuscitation, we only modelled a QALY impact on the child based on studies that showed that there was no HRQoL impact to these mothers [26]. In our sensitivity scenarios we considered the impact on the mothers of foetal death.

We calculated the impact of PHTs/no transfusions over the whole of one cohort's lifetime and expressed this in two sets of outcome measures (Figure 1). The first set of outcome measures was split by health state and included: (i) the number of patients ending up in each health state; (ii) the total QALYs for all patients in each health state in a given year, and; (iii) the discounted monetized value of QALYs for each health state. These enabled us to calculate a second set of aggregated measures: (i) the summed total of QALYs across all health states; (ii) the summed value of discounted monetized QALYs across all health states, and; (iii) the summed total of QALYs across all health states per person.

Modelling impact on babies born to patients who suffered trauma

When RhD-positive RBCs were transfused, we calculated the potential impact on babies born with HDFN or on those who died because of HDFN. Whereas the specific harms to patients were expressed as the QALYs attributed to patients in each health state, the harms to babies dying from HDFN or experiencing a long-term adverse event from it were expressed as the QALYs lost, compared to if those babies had not suffered HDFN. This QALY difference was subtracted from

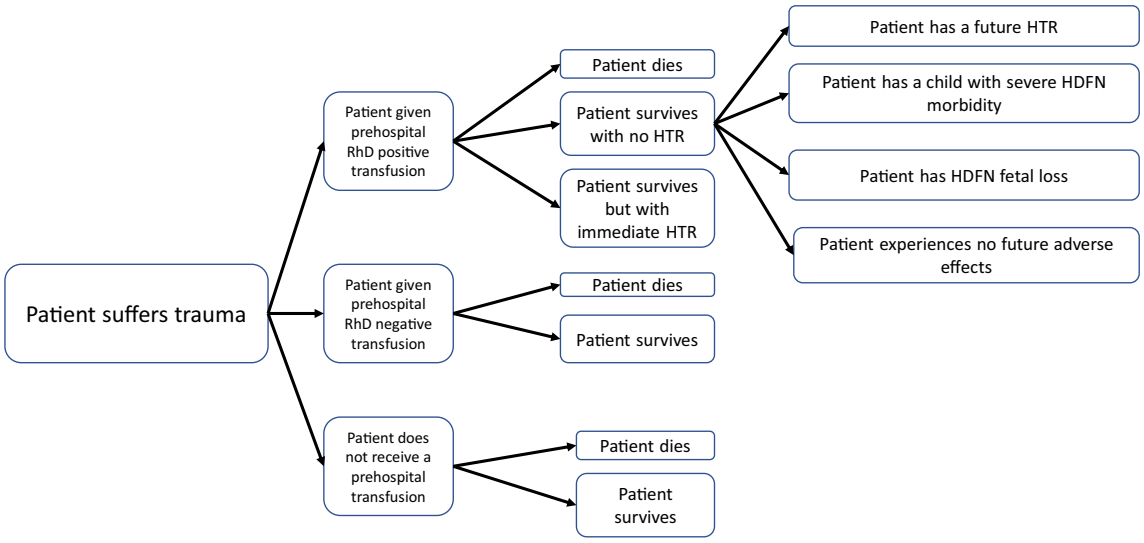

FIGURE 1 Pathway of possible patient outcomes following trauma and PHT. HDFN, haemolytic disease of the foetus and newborn; HTR, haemolytic transfusion reaction

TABLE 1 Outcomes of the entire cohort of injured patients ($n = 5561$) stratified by the RhD-type of prehospital RBCs transfused

Outcomes	Prehospital RhD-negative RBC recipients			Prehospital RhD-positive RBC recipients			No prehospital RBC transfusion		
	No. of patients	Total QALY per person over lifetime	Total QALYs in entire population	No. of patients	Total QALY per person over lifetime	Total QALYs in entire population	No. of patients	Total QALY per person over lifetime	Total QALYs in entire population
Patient survives trauma without harm	4927	28.8	141,899	4926.5	28.8	141,883	4360	27.4	119,285
Patient dies in trauma	634	0	0	634	0	0	1201	0	0
Patient survives trauma but experiences HTR morbidity	0	0	0	0.19	28.7	5	0	0	0
Patient survives trauma but dies from HTR	0	0	0	0.02	0	0	0	0	0
Patient survives trauma but has a child with severe HDFN morbidity	0	0	0	0.13	17.8	3	0	0	0
Patient survives trauma but has a child with HDFN mortality	0	0	0	0.18	28.8	5	0	0	0
Foetus/neonate experiences major morbidity from HDFN	0	0	0	0.13	NA	−4.8	0	0	0
Foetus/neonate dies from HDFN	0	0	0	0.18	NA	−12.4	0	0	0
Total	5561		141,899	5561		141,879.8	5561		119,285
Discounted QALYs			102,147			102,135			85,868
Discounted QALYs per patient			18.368			18.367			15.441
Discounted monetized QALYs			£7,150,296,980			£7,149,599,392			£6,010,768,839
Discounted monetized QALYs per patient			£1,285,793			£1,285,668			£1,080,879

Abbreviations: HDFN, haemolytic disease of the foetus and newborn; HTR, haemolytic transfusion reaction; QALY, quality-adjusted life year; RBC, red blood cell.

TABLE 2 Outcomes of the subset cohort of injured women <50 years old ($n = 100$) stratified by the RhD-type of prehospital RBCs transfused

Outcome	Prehospital RhD-negative RBC recipients			Prehospital RhD-positive RBC recipients			No prehospital RBC transfusion		
	No. of patients	Total QALY per person over lifetime	Total QALYs in entire population	No. of patients	Total QALY per person over lifetime	Total QALYs in entire population	No. of patients	Total QALY per person over lifetime	Total QALYs in entire population
Patient survives trauma without harm	88.6	33.6	2977	88.3	33.6	2966	78.4	31.9	2503
Patient dies in trauma	11.4	0	0	11.4	0	0	21.6	0	0
Patient survives trauma but experiences HTR morbidity	0	0	0	0.01	33.5	1	0	0	0
Patient survives trauma but has a child with severe HDFN morbidity	0	0	0	0.001	0	0	0	0	0
Patient survives trauma but has a child with HDFN mortality	0	0	0	0.13	20.7	3	0	0	0
Foetus/neonate dies from HDFN	0	0	0	0.17	33.6	6	0	0	0
Foetus/neonate experiences major morbidity from HDFN	0	0	0	0.13	NA	−4.9	0	0	0
Foetus/neonate dies from HDFN	0	0	0	0.17	NA	−12.3	0	0	0
Total	100		2977	5561		2958.8	100		2503
Discounted QALYs			2035			2025			1710
Discounted QALYs per patient			20.345			20.254			17.103
Discounted monetized QALYs			£142,415,783			£141,778,854			£119,719,272
Discounted monetized QALYs per patient			£1,424,158			£1,417,789			£1,197,193

Abbreviations: HDFN, haemolytic disease of the foetus and newborn; HTR, haemolytic transfusion reaction; QALY, quality-adjusted life year; RBC, red blood cell.

TABLE 3 Sensitivity analysis performed on the entire cohort of injured patients ($n = 5561$) and on the subset cohort of injured women <50 years old ($n = 667$)

	Overall cohort ($n = 5561$)				Subset analysis of women ≤50 ($n = 100$)			
	Main analysis	Sensitivity analysis	Total lifetime QALYs whole population—main	Total lifetime QALYs whole population—sensitivity	Main analysis	Sensitivity analysis	Total lifetime QALYs whole population—main	Total lifetime QALYs whole population—sensitivity
(A) QoL parameter								
QoL of child post HDFN	0.56	0.47			0.56	0.47		
QoL of mother following foetus/neonatal HDFN death	0.6	0.3			0.6	0.3		
QoL of mother of HDFN child with disabilities	0.37	0.3			0.37	0.3		
Total			141,880	141,876			2958	2954
(B) Harms' probabilities								
Probability of immediate HTR	0.004%	0.025%			0.016%	0.12%		
Probability of future HTR	0.00021%	0.0006%			0.0014%	0.024%		
Average number of babies per (surviving) patient	0.10	0.14			0.81	0.99		
Probability that baby is at risk of HDFN	2.34%	4.9600%			15.60%	31.02%		
Probability that an at-risk baby dies from HDFN	0.015%	0.023%			0.015%	0.023%		
Probability that an at-risk baby has severe disability from HDFN	0.012%	0.017%			0.012%	0.02%		
Total			141,880	141,813			2958	2909
(C) 24-h survival rate (%)								
Prehospital RhD −ve RBC transfusion	88.6%	81.4%	141,899	130,368	88.6%	81.4%	2977	2735
Prehospital RhD +ve RBC transfusion	88.6%	81.4%	141,880	130,350	88.6%	81.4%	2958	2718
No PHT	78.4%	78.4%	119,285	119,285	78.4%	78.4%	2503	2503
All sensitivities changed								
Prehospital RhD −ve RBC transfusion				130,368				2735
Prehospital RhD +ve RBC transfusion				130,274				2659
No PHT				119,285				2503

Note: The main analysis refers to the analysis that was performed using the values described in the methods; the sensitivity analysis refers to the analysis performed using the values listed for the three parameters in this table.
Abbreviations: HDFN, haemolytic disease of the foetus and newborn; HRQoL, health-related quality of life; HTR, haemolytic transfusion reaction; QALY, quality-adjusted life year; RBC, red blood cell.

the total QALYs experienced by the cohort. Key Modelling Assumptions made are described under Supporting information.

Sensitivity analysis

The sensitivity analysis modified three sets of parameters to test 'worst-case' scenarios: (i) HRQoL measures, (ii) probabilities of harms leading to the three risks, and (iii) trauma survival rates. Overall, five scenarios were modelled for each policy option: the main scenario, three sensitivity scenarios changing the parameter groups above individually, and one sensitivity scenario changing all parameters simultaneously.

RESULTS

Based on survival data from studies of patients who received PHTs [1, 2], we calculated that an estimated 5561 injured patients per year in England who received PHTs, 634/5561 (11.4%) would die within the first 24 h. Patients who received RhD-negative RBCs would not have experienced immediate or delayed HTRs caused by anti-D or be at risk for future HDFN. The total number of QALYs over the lifetime of the recipients of prehospital RhD-negative RBCs who survived the trauma was calculated to be 141,899 (Table 1).

For patients who received RhD-positive RBCs, the same percentage would be expected to die at 24 h because both RhD-positive and RhD-negative RBCs were assumed to confer the same survival benefits. However, due to receipt of RhD-positive RBCs, the model predicted that, of these survivors, there would be a small number of patients who would directly experience any anti-D mediated adverse events. In addition, the receipt of the RhD-positive RBCs would be expected to cause 0.13 foetal/neonatal major morbidity events and 0.18 foetal/neonatal mortality events due to HDFN caused by transfusing RhD-positive RBCs to the female trauma patients in this cohort. These foetal/neonatal adverse events reduce the total number of QALYs among the recipients of RhD-positive prehospital RBCs to 141,879.8, a difference of 19.2 QALYs for the entire cohort compared to recipients of prehospital RhD-negative RBCs, with the monetization value being almost £600,000.

For the group of patients who did not receive a PHT, the model predicted that there would be 1201/5561 (21.6%) deaths leading to approximately 22,600 fewer QALYs over the lifetime of the recipients in this group (119,285) compared to those who received a PHT. There were no other anti-D mediated adverse events predicted to occur in this group, as they were not exposed to the RhD-antigen through PHT. Using the valuation approach outlined in the Green Book [27], a publication from the United Kingdom Treasury that provides guidance on how to appraise policies, programmes and projects, the resulting loss of QALYs from not providing RBCs for prehospital resuscitation would be valued at just over £1billion compared to providing RBCs of any RhD type over the lifetime of these patients (Table 1).

Of the 5561 patients in England who received a PHT, 100 would have been FCPs who were at risk of a future pregnancy affected by HDFN [15]. The provision of RhD-positive RBCs would be expected to lead to very few patients in this cohort experiencing any of the anti-D mediated adverse events, and there would be 0.13 foetal/neonatal major morbidity events and 0.17 foetal/neonatal mortality events per year due to HDFN caused by transfusing RhD-positive RBCs to this group. Because of the small number of adverse events caused by using RhD-positive RBCs, the total number of QALYs among these recipients would be reduced by only 18.2 compared to the group that received RhD-negative RBCs (2977 vs. 2958.8, respectively). Not providing RBCs for prehospital resuscitation would diminish the total number of QALYs for FCPs by approximately 470 compared to RBC recipients due to the smaller number of expected survivors among the non-transfused patients. For these FCPs, not providing prehospital RBCs would result in a QALY loss worth approximately £22.7 million compared to providing RBCs of any RhD type over the lifetime of these patients (Table 2).

Sensitivity analyses

Sensitivity analyses, given a degree of uncertainty relating to some of the key model inputs, were performed by adjusting some of these parameters (Table 3). Changing the HRQoL parameters caused a reduction of only four QALYs in the overall (0.00%) and the subset (0.14%) cohorts when RhD-positive blood was used. This is mainly from considering an HRQoL impact on a mother experiencing foetal death which was not considered in the main scenario. Similarly, the difference in the total number of lifetime QALYs for the probability of experiencing an adverse event between the main and the sensitivity analyses of the overall cohort was very small (0.05%; 67 QALYs), with a slightly higher difference (1.67%; 49 QALYs) observed in the analysis of the subset of FCP.

The third sensitivity scenario reduced the survival benefits from 10.2% to 3% (compared to no transfusion), based on the effect of PHTs on episode mortality observed in the RePHILL trial [6]. With lower survival rates, fewer QALYs were gained regardless of the RhD nature of the transfused blood. However, even with reduced survival benefits, prehospital RBC transfusion of either RhD-type continued to be advantageous compared to no transfusion. Survival increases of only 0.02% for the entire cohort and 0.7% for the subset cohort from providing PHTs would be sufficient to make an RhD-positive strategy better in terms of QALYs than not providing PHTs. Changing all sensitivity parameters simultaneously still showed that prehospital RBC transfusion offers greater QALYs gained compared to not providing PHTs, even if RhD-positive blood is used.

DISCUSSION

Previous models of transfusing RhD-positive RBCs to injured trauma patients of unknown RhD-type have presented the risks of adverse

events as probabilities of their occurrence [12–14]. This study is unique because it quantified the relative benefit of PHT in terms of QALYs gained, which provides another means of comparing different approaches to implementing a PHT program. Our results show that while the use of RhD-positive RBCs carries small risks, the benefits measured in QALYs are substantially larger than if no PHTs are administered, even for RhD-negative FCPs. This suggests that if RhD-negative RBCs are unavailable, the use of RhD-positive RBCs is a preferable alternative to not providing any prehospital RBC transfusions.

This result is driven by the survival benefits of providing PHTs, while the likelihood of RhD-positive RBCs causing harm is comparatively very low. In our model, we assumed a survival benefit of 10.2% based on two observational studies [1, 2]; however, there is uncertainty about the magnitude of survival benefit from providing PHTs. The sensitivity analysis of the model demonstrated that even when we lowered the survival benefit to 3% in line with episode mortality findings from the RePHILL trial [6]—RhD-positive RBCs are preferable to not providing a PHT.

Following the United Kingdom government Treasury green book [27] approach for valuing QALYs, a PHT provides approximately £205,000 worth of value per patient on average, that is, the difference in discounted monetized QALYs per patient receiving RhD-positive or negative blood compared with no transfusion. The NHS cost of supplying a unit of RBCs is £153.10. The cost of administering an RBC transfusion is estimated as £57 for the first unit and £36 for subsequent units [28]. Therefore, the cost for a patient receiving two units of RBCs prehospital, which is the current average amount transfused in the prehospital setting from a recent survey of emergency medical services in the United Kingdom (LG personal communication), is about £400. This is substantially lower than the value of the QALY benefit. PHTs provide 2.93 discounted QALYs per patient, which at the cost of £400, is the cost per QALY of £137 and is substantially below the NICE guide of £20,000–30,000 per QALY.

Comparing the discounted monetized QALYs per patient between recipients of RhD-negative RBCs and RhD-positive RBCs, the use of RhD-negative blood provides about £125 more value per patient than RhD-positive blood. However, the equivalent figure for RhD-negative FCPs is £6369, indicating that a policy of providing RhD-negative blood for FCPs provides benefits and should be pursued where possible. Hospitals in England currently pay the same amount for RhD-negative and RhD-positive group O blood. However, RhD-negative blood is in higher demand, and blood services spend additional resources to recruit and retain more RhD-negative donors. The totality of these costs is not well described in the literature, but it seems unlikely to exceed the value of providing RhD-negative blood to FCPs. Furthermore, it is less clear what the totality of these costs will be for the whole patient group and whether recruitment costs will outweigh patient benefits, and further research is required to understand these.

This study has several limitations. First, since the model was built using data from the literature, the extent to which these findings can be generalizable depends on the generalizability of the original data. Secondly, it would have been good to have provided a projection on the effect of blood transfusion on QALYs based on the patient's injury severity; however, all studies/trials have pooled the transfusion effect for all types and injury severity, and we were not able to quantify this in QALY terms. Thirdly, this study was designed to describe the effect of PHT on the 24-h mortality rate, as this time-point is the most likely to be modifiable by PHT, as beyond the 24 h, the cause of death in trauma patients is typically factors other than exsanguination. Indeed, the American National Heart, Lung, and Blood Institute and Department of Defence have recently supported the development of short-term outcomes (3–6 h mortality) for adult trauma patients based on recent RCTs [29]. In the United Kingdom, the RCT of whole blood versus standard of care in the prehospital setting, will use the composite of mortality or massive transfusion at 24 h as its primary outcome. Therefore, the use of 24 h mortality in this model took all these development into consideration.

In conclusion, this study quantified the change in QALYs based on two PHT strategies compared to not providing PHTs to injured patients. While a small number of patients were predicted to experience harm from receiving RhD-positive RBCs, the number of QALYs gained by providing prehospital RBCs was much larger. Our data support a policy of using RhD-negative blood for FCPs where possible. The quantification of transfusion risks and benefits prehospital in terms of QALY also enables future work to compare the allocation of group O RhD-negative blood between different clinical settings to maximize health outcomes and to consider cost-effectiveness.

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CONFLICT OF INTEREST

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SUPPORTING INFORMATION

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

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ORIGINAL ARTICLE

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Single-exon approach to non-invasive fetal *RHD* screening in early pregnancy: An update after 10 years' experience

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Abstract

Background and Objectives: Anti-D prophylaxis, administered to RhD-negative women, has significantly reduced the incidence of RhD immunization. Non-invasive fetal *RHD* screening has been used in Stockholm for more than 10 years to identify women who will benefit from prophylaxis. The method is based on a single-exon approach and is used in early pregnancy. The aim of this study was to update the performance of the method.

Materials and Methods: The single exon assay from Devyser AB is a multiplex kit detecting both exon 4 of the *RHD* gene and the housekeeping gene *GAPDH*. Cell-free DNA was extracted from 1 ml of plasma from EDTA blood taken during early pregnancy, weeks 10–12. The genetic *RHD* results were compared with serological typing of newborns for a determination of sensitivity and specificity.

Results: In total, 4337 pregnancies were included in the study; 44 samples (1%) were inconclusive either due to maternal *RHD* gene variants ($n = 34$) or technical reasons ($n = 10$). Of the remaining 4293 pregnancies, a total number of nine discrepant results were found. False positive results ($n = 7$) were mainly ($n = 4$) due to *RHD* gene variants in the child. False-negative results were found in two cases, of which one was caused by a technical error. None of the false-negative cases was due to *RHD* gene variants. Overall, the sensitivity of the method was 99.93% and specificity 99.56%.

Conclusion: The single-exon assay used in this study is correlated with high sensitivity and specificity.

Keywords

fetal *RHD*, NIPT *RHD*, single-exon

Highlights

- Assay sensitivity was 99.93% and specificity 99.56%.
- The single-exon approach is safe and accurate for fetal *RHD* screening.

INTRODUCTION

Haemolytic disease of the fetus and newborn (HDFN) is caused by maternal alloantibodies directed against fetal antigens on the red cell surface [1]. The RhD antigen is most frequently involved in HDFN, and therefore, potentially life threatening for the fetus. Antenatal and postpartum anti-D prophylaxis has reduced the incidence of RhD immunization to low levels, 0.2%–0.4% [2–4]. Previously, antenatal anti-D was often administered to all RhD-negative women, resulting in unnecessary treatment of those, approximately 40%, carrying an RhD-negative fetus. In an increasing number of countries, antenatal anti-D immunoglobin is used as prophylaxis only in RhD-negative women with an *RHD*-positive fetus [5].

Non-invasive prenatal test (NIPT) for fetal *RHD* has been used for more than 20 years. Initially, it was used to determine the fetal RhD status in immunized pregnancies but is now an established screening method in many laboratories to identify women who will benefit from prophylaxis [6–9]. The methodology is based on the purification of free circulating DNA from plasma samples and subsequent PCR amplification of one or several *RHD*-specific exons. It is essential that a fetal RhD assay has a high diagnostic sensitivity to avoid false-negative results, which may result in missed anti-D prophylaxis and risk of immunization. A positive control to estimate the amount of fetal DNA in the sample is desired, but it has been challenging to find suitable controls, and therefore, most assays lack a specific control for fetal DNA [10]. For DNA purification, virus isolation kits or specific kits for circulating DNA are used. For PCR analysis, different target exons and combinations of exons can be used in most available real-time instruments. Until recently, most assays have been using in-house methods; however, several CE-approved commercial kits for *RHD* PCR amplification are now available.

In Stockholm, a screening study for NIPT of fetal *RHD* in combination with targeted antenatal anti-D prophylaxis was introduced in 2009, and the first evaluation was published in 2012 [11]. Using a single-exon approach in early pregnancy, we showed that the assay was safe and accurate for samples obtained after week 8. In a clinical follow-up study, we showed that the immunization rate was reduced to a comparable level, 0.26%, to that in programs providing antenatal prophylaxis to all RhD-negative women [12]. Since then, routine blood sampling have been recommended after gestational week 10, and most samples are taken during gestational weeks 10–12. Since then, a number of changes in the procedure have been implemented, for example, extended automation and minor technical modifications and improvements in the method. Before abolishing routine serological typing of newborns, we decided to perform a new validation.

MATERIALS AND METHODS

Included samples

Samples from pregnant RhD-negative women analysed from May 2019 to October 2020 were included.

Whole blood samples, 5 or 7 ml EDTA tubes, were collected at antenatal or blood sampling centres and sent to the laboratory by regular post or transportation service. Only blood samples no older than 5 days were accepted for analysis. The samples were centrifuged for 15 min at 1500*g*. The blood tubes, were in most cases, put directly onto the QIAsymphony (QIAGEN, Hilden, Germany) instrument for DNA extraction but for samples close to 5 days before they could be analysed, plasma was transferred to cryotubes and frozen at −20°C until further analysis. As shown by others, the use of short-time frozen plasma does not have a major effect on results [13].

DNA extraction and PCR analysis

DNA extraction was performed using the QIAsymphony instrument in combination with the Virus/Pathogen kit (QIAGEN, Hilden, Germany). For each sample, 1 ml plasma was used for extraction, and the elution volume was 85 µl. A total of 24 samples were extracted in each run. DNA samples were usually stored in the freezer at −20°C until PCR analysis, since samples were needed to be collected for a total number of 31 before PCR analysis. The storage time at −20°C was less than 3 days.

PCR analysis was performed using a CE-labelled single exon kit (Devyser, Stockholm, Sweden). The PCR assay is designed for multiplex detection of *RHD* exon 4 and the endogenous control gene *GAPDH*. In May 2019, the composition of the PCR kit was updated to improve the signal intensity and decrease background signals (communication with Devyser).

For PCR analysis, 31 samples in triplicate + positive and non-template control controls were run in a 96-well PCR plate using the ABI 7500 Real-Time PCR instrument. The total PCR volume was 50 µl and consisted of 30 µl of PCR-mix + 20 µl of the extracted DNA sample. The PCR program was 95°C for 10 min and 50 cycles of 95°C for 15 s and 62°C for 1 min.

The plasma-equivalent per PCR reaction was 235 µl (1000 µl * 20 µl/85 µl).

For a positive result, at least two-thirds of replicates should be positive. Samples that were positive in one-third of replicates were reanalyzed if an extra aliquot of plasma was available; otherwise, a new sample was requested. For *RHD*-negative samples, the Ct values of the *GAPDH* gene needed to be in a specific range as determined by the manufacturer for the specific PCR-kit batch. Too low or too high *GAPDH* Ct-values indicated the presence of too high maternal DNA concentration or too low total-DNA concentration, respectively. In samples with too high *GAPDH* Ct-value, a reanalysis was performed if an extra aliquot of plasma was available, but in samples with too low *GAPDH* Ct-value, a new sample was requested directly. After two consecutive inconclusive results, no additional samples were requested, and anti-D-prophylaxis was recommended. The specific Ct value ranges for *GAPDH* used to approve samples correspond to approximately 150 (lower limit) to 40.000 (higher limit) genomic equivalents per 1 ml plasma.

For *RHD*-negative samples that showed a strong *RHD* signal, that is, Ct value for *RHD* was lower than the Ct value for *GAPDH*, a maternal *RHD* variant was suspected. These samples were further investigated for *RHD* variants with the Fluogene genomic typing system (Innotrain, Kronberg, Germany). Samples with unclear results were also analysed with an in-house developed NGS method for blood group typing (not published). In short, the NGS method was designed to analyse the most common blood groups by sequencing only the most informative exons. For *RHD* and *RHCE*, all 10 exons were sequenced, including intron sequences in the junction. *RHD* zygosity was determined by copy number variation analysis.

Cord blood type

ABO and RhD blood typing was performed after birth on EDTA-anticoagulated umbilical cord blood samples or citrate samples from the newborns. Blood typing was done in the automated system Vision using the BioVue cassettes ABO-Rh/DAT (Ortho Clinical Diagnostics Raritan, NJ, USA). The blood group serology results were used as the gold standard to assess the performance of the antenatal fetal *RHD* genotyping.

Sensitivity and specificity calculations

For determination of diagnostic sensitivity and specificity, genetic fetal *RHD* typing was compared to serological typing performed at birth. Sensitivity was calculated as a proportion of true PCR positives in comparison to serology of the newborn = true positive/(true positive + false-negative).

Specificity was calculated as a proportion of true PCR negatives in comparison to serology of the newborn = true negative/(true negative + false-positive).

The study was approved by the Stockholm Regional Ethics Committee (no 2022-01282-02).

RESULTS

Samples

In total, 7579 samples in 7066 pregnancies were analysed. After exclusion of samples from pregnancies outside Stockholm, terminated pregnancies and pregnancies with missing blood group serology results in the newborn, 4337 pregnancies were included in this study. After a first analysis, a total number of 151 samples (3.5%) showed inconclusive results. Of these samples, maternal *RHD* variants were suspected in 34 cases. Of the remaining 117 samples, the reason for inconclusive results were one-third positive replicates in 49 samples (42%), too low *GAPDH* Ct-value in 44 samples (38%) or too high *GAPDH* Ct-value in 24 samples (20%). After reanalysis of the extra aliquot of plasma or resampling, conclusive results were obtained for most samples, but in 10 pregnancies (0.2%) the results remained inconclusive (Figure 1). The reasons for inconclusive results for these 10 cases were no re-sampling ($n = 7$), too low *GAPDH* Ct-value ($n = 2$) or too high *GAPDH* Ct-value ($n = 1$). The RhD status of these 10 newborns was positive in two cases (both in the group of '1/3 pos') and negative in eight cases.

Excluding the 34 pregnancies with suspicion of maternal *RHD* variants and the 10 pregnancies with inconclusive results, a total number of 4293 pregnancies were included for determination of sensitivity and specificity.

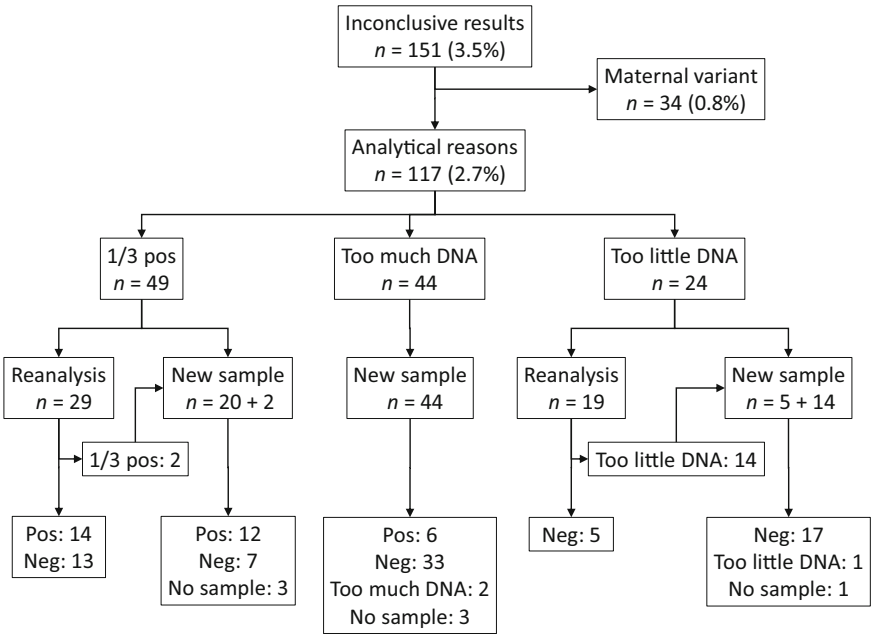

FIGURE 1 Reasons and outcome for inconclusive results

Sensitivity and specificity

Of the 4293 pregnancies, 2692 showed *RHD*-positive results and 1601 *RHD*-negative results. A total number of nine discrepant cases were found when comparing fetal *RHD* genotyping and serological typing at birth (Figure 2).

False-positive results were detected in seven cases (0.16%). In four out of seven cases, *RHD*-gene variants were found in the children after genotyping (two with RHD*Psi, one with K409K and one with c.635-1G>A). In two other cases, maternal *RHD* variants were suspected since the children lacked the *RHD* gene when genotyped. In both cases, the *RHD* signal was strong at the time of fetal *RHD* analysis but not strong enough to report as a suspected maternal *RHD* gene. Unfortunately, no sample was available from the mothers for genotyping. The last case showed a positive *RHD* signal in two-thirds of replicates at the time of analysis. This is the only case that shows a 'real' false-positive result caused by the analysis and not due to *RHD* gene variants.

False-negative results were detected in two cases. One case was probably caused by insufficient mixing of PCR-mix. Amplification curves were not optimal, and the RHD signals were not strong enough to cross the threshold for a positive result. In the other case, the total amount of DNA was lower than normal, Ct-value for *GAPDH* was high but still within the accepted range. It could be that a combination of low DNA amount and a low fraction of fetal DNA made it difficult to detect fetal *RHD* in this sample.

Overall, the sensitivity of the method was 99.93% (95% CI 99.73%–99.99%) and specificity 99.56% (95% CI 99.08%–99.82%).

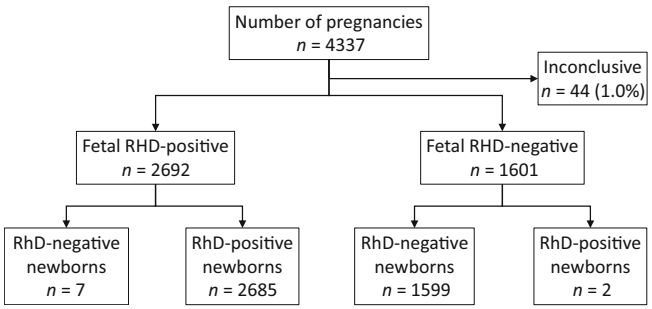

FIGURE 2 Summary of the results. Genomic versus serological typing

Maternal RHD variants

In 34 cases, the *RHD* status of the fetus could not be determined due to the suspicion of a maternal *RHD* variant. These cases were further analysed with genomic typing if possible. In Table 1, the results of the genomic typings are shown. The most common variant was RHD*Psi followed by DAU. A variant, not previously described, was found in one case. This was a frameshift mutation caused by a 1-bp deletion at position c.1178 (or c.1179) in exon 9. In two cases, analysis of maternal DNA detected no *RHD* gene. In both cases, the fraction of fetal DNA was likely high enough to produce a strong *RHD* signal.

DISCUSSION

In this study, we have evaluated the performance of a single-exon screening assay for non-invasive fetal *RHD* determination during early pregnancy, weeks 10–12. In the previous study from 2012, we observed a sensitivity of 99.3% when excluding samples before gestational week 10 [11]. Sensitivity was 99.93% in the current study. During the past 10 years we have gained better experience in running the tests, stepwise improvements have been made in the procedures, and instruments and the *RHD* assay has been optimized.

The method we have used is a single-exon assay for the detection of *RHD* exon 4 in combination with the housekeeping gene *GAPDH*. In many other studies, a combination of different exons, for example, exons 4, 5, 7 and 10, have been used [6, 8, 14]. The major advantage of using several exons is to minimize the risk of false-negative results due to *RHD* variants. However, as shown from this and our previous

TABLE 1 Summary of pregnancies with suspicion of maternal *RHD* variants ($n = 34$)

RHD change	*n*	DNA changes	RhD consequence	ISBT allele name	*RHCE*-alleles
RHD*Psi	8	Several	Null allele	RHD*08N.01	ce
DAU	5	c.1136C>T	p.Thr379Met	RHD*10	ce
Weak 11	4	c.885G>T	p.Met295Ile	RHD*11	Cce
Weak 15	3	c.845G>A	p.Gly282Asp	RHD*15	cEe
RHD-RHCE(3)-weak D type 4.0	2	Exon 3 hybrid on a weak 4 background	Null allele	RHD*01N.72	ce
Weak 2	1	c.1154G>C	p.Gly385Ala	RHD*01W.2	CcEe
Weak 92	1	c.1145T>C	p.Leu382Pro	RHD*01W.92	Cce
New	1	c.1178delG (or 1179delG)	Frameshift mutation	—	Cce
Not known[a]	5				
Not analysed	2				
No RHD gene found	2				

[a]RHD gene detected but not further analysed for variants.

study, false-negative results have usually been due to technical reasons and not at all due to undetected *RHD* variants in the fetus. A sensitivity of 99.93% also shows the accuracy of this method. This could, of course, reflect the population included in the study and may differ in other populations where other *RHD* variants are present, although today, we have a diverse population in the Stockholm area, with many ethnicities represented [15–17].

Another advantage of using the multi-exon approach is that maternal *RHD* variants like RHD psi can be identified if one of the exons is designed not to amplify this variant. In these cases, the RHD status of the fetus can be determined, and the number of inconclusive and false-positive results may be reduced.

One of the false-negative results was probably caused by low amount of free circulating DNA. The amount of free circulating DNA, both total and fetal DNA fraction, usually varies from one individual to another. In addition, different factors, for example, body weight, can affect the DNA amount [18, 19]. Also, lysis of the blood during shipping and/or storage can cause leakage of genomic DNA from the mother and decrease the sensitivity of the method. Using an internal control gene like *GAPDH* can provide information about the total amount of DNA present and serve as an indicator of the quality of the sample. In six samples, the first result showed a negative result, but the test was considered inconclusive since the *GAPDH* signal indicated too much DNA. Re-sampling of these cases showed positive results. Without the presence of an internal control gene, these samples would have been reported as false-negative.

In samples with a high Ct value for *GAPDH* (too little DNA), a re-analysis of the extra aliquot of plasma showed inconclusive results again in 14/19 samples. New samples were requested for these patients. Because of low success rates in the re-analysis of the same sample, we have decided to request a new sample directly for these patients.

False-positive results were found in seven cases, and four of them were due to *RHD* variants in the fetus. In two cases, probably maternal *RHD* variants caused false-positive results. The diagnosis of maternal *RHD* variants is based on the signal strengths from *RHD* versus *GAPDH* and may not always be 100% accurate. This was also shown in two other cases where maternal *RHD* variants were suspected, but further investigation showed that the signals were likely due to high fetal-DNA content in the sample. Whether it is an *RHD* variant in the fetus or mother does not matter for clinical decision-making since anti-D prophylaxis is recommended in both cases. Altogether the rate of false-positive results was 0.16%, which means that a relatively small number of pregnant women are administered unnecessary anti-D prophylaxis. In addition, 34 women (0.78%) were given anti-D prophylaxis since the genotype of the fetus could not be determined due to a possible maternal *RHD* variant.

The quality of the blood sample and the extracted DNA is of utmost importance for reliable analysis of fetal *RHD*. The best approach is a fully automated workflow and minimizing freeze-thawing of plasma and/or DNA samples [20, 21]. In this study, we worked mainly on fresh plasma samples from primary blood tubes. However, since the number of samples used for DNA extraction using QIAsymphony ($n = 24$) did not match with the previous size of the PCR kit ($n = 31$), we needed to freeze DNA samples in order to sum up to 31 samples before PCR analysis. Since the completion of this study, the PCR kit format has been updated for the analysis of 24 samples, and the QIAsymphony instrument has been upgraded with an AS module for PCR setup. The workflow from sample preparation to PCR assay setup is now fully automated. We have already seen effects on the number of inconclusive samples. The fraction of inconclusive results due to analytical reasons is now around 2% as compared to previously 3.0%–3.5%.

In summary, the single-exon approach used in this study for fetal *RHD* determination during early pregnancy is correlated with high sensitivity and specificity. As of November 2020, we have discontinued serological typing of newborns.

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CONFLICT OF INTEREST

Mehmet Uzunel was employed by Karolinska University Hospital when this study was initiated but is currently working at Devyser AB. Eleonor Tiblad, Anette Mörtberg and Agneta Wikman do not have any conflicts of interest/competing interests to declare.

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ORIGINAL ARTICLE

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Laser incubation for the rapid detection of red cell alloantibodies in human blood samples

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Abstract

Background and Objectives: Pre-transfusion antibody screening requires the detection and identification of immunoglobulin G (IgG) antibodies against red blood cells (RBCs). Using the indirect antiglobulin test (IAT), plasma–RBC solutions are incubated at 37°C in gel cards, typically by heating block technology. Here, we apply the newly developed laser incubation method to detect RBC alloantibodies in the plasma from human donors.

Materials and Methods: Donated human plasma samples ($N = 128$) containing clinically significant IgG antibodies directed against Rh (D, C, c, C^w and E), Kell (K and Kp^a), Duffy (Fy^a and Fy^b), Kidd (Jk^a) and MNS (S) blood group system antigens were tested by the indirect antiglobulin test (IAT). Samples were heated to 37°C by infrared laser (980 nm) for incubations of up to 5 min. Samples were also incubated in a heating block for comparison.

Results: When heating by laser, the presence of an alloantibody is detected after only a 1-min incubation for 96% of samples. No samples required longer than 3 min of laser incubation in order to detect the antibody. For all samples, incubation by laser gave the same or stronger result within 5 min. No samples required longer than 5 min to achieve an equivalent result to that of the 5-min heating block incubation. The laser was not found to damage cells or antibodies.

Conclusion: Laser incubation provides comparable results in shorter time frames than the heating block. Laser incubation can rapidly detect even very weak antibodies.

Keywords

alloantibodies, antibody–antigen complex, IAT, incubation, laser, red blood cell

Highlights

- The newly developed method of heating blood samples by laser is for the first time applied to human plasma samples.
- Red cell alloantibodies from a range of blood group systems are detected and correctly identified.
- Detection is rapid, taking 3 min of laser incubation or less time for all samples.

INTRODUCTION

Detection of alloantibodies against red blood cells (RBCs) is a critical part of pre-transfusion compatibility testing and antenatal screening. The indirect antiglobulin test (IAT) is required to identify the presence of immunoglobulin G (IgG) antibodies that are active at 37°C and considered to be clinically significant, including those responsible for haemolytic transfusion reactions and haemolytic disease of the foetus and newborn. Accordingly, the IAT involves an incubation phase at 37°C, a practice that has been in place since the test's conception in the 1940s [1], which has since undergone surprisingly little investigation [2–6].

Testing for IgG antibodies is labour-intensive and time-consuming; it may induce human error and delay the provision of compatible blood for urgent transfusions. Firstly, patient plasma is screened for the presence of any antibody by testing against a set of RBCs that are positive for a range of key antigens. If positive, the plasma is then tested against a panel of RBCs to identify the antibody. For both stages of this two-step process, the plasma–RBC solution must first be separately heated to 37°C. This heating step both enhances clinically significant IgG antibodies and mitigates room temperature reactions, which would otherwise provide false positives.

The antibody–antigen complexation reaction requires thermal activation, and the mechanism involves a set of reversible reactions with competing, minute-scale association and dissociation rates [7, 8]. For each antigen–antibody complex, in time and given the right temperature, an equilibrium is reached where binding is maximum [9]. However, the equilibrium dependence on incubation time and temperature for antigen–antibody complexes from different blood group systems remains poorly understood and in parts contradictory [2–6].

Traditional incubation methods for the IAT rely on water baths or ovens, and could take up to an hour [2]. With the use of low ionic strength solution (LISS), and the recent use of gel card technology and dedicated gel card heating blocks, incubation time for IAT has been reduced to less than 15 min [5, 10–12]. However, the procedure still requires two separate steps: incubation and centrifugation of the gel cards, which uses two different stations requiring manual transfer of the gel cards between them. This could increase the time delay and can introduce inconsistencies due to human error.

Laser incubation for immunohaematological testing has been demonstrated to be a potential method for rapid antibody screening [6, 13], which could reduce time delays for emergency blood transfusions. The speed of laser incubation is due to the selective photothermal response of haemoglobin to the laser light, meaning that the RBC surface is preferentially heated at the binding point of the antigen and antibody [6]. Using near-infrared (980 nm) laser light to photothermally heat solutions, samples can be heated up to 37°C in less than 30 s. In a previous study, samples containing reagent RBCs were tested against anti-D reagent antiserum. For incubation time less than 1 min, clear positive agglutination results were obtained with no false positives, no damage to RBCs or antibodies, and no destruction or dissociation of agglutinates [6]. However, the use of laser incubation on plasma-borne antibodies in clinical samples was not tested, which is the focus of this study.

We now report the results from the application of laser incubation of CAT gel cards for a wide range of clinically significant plasma-borne alloantibodies ($N = 128$) from the Rh (D, C, c, C^w and E), Kell (K and Kp^a), Duffy (Fy^a and Fy^b), Kidd (Jk^a) and MNS (S) blood group systems. It is our objective to demonstrate that laser incubation is a viable option for the rapid testing of patient samples for antibody screening and identification, that laser incubation produces comparable results to current IAT methods in shorter time frames, and that significant simplification of IAT equipment by incorporation of laser incubation is now possible.

MATERIALS AND METHODS

Test population

From 104 donors, 128 alloantibody samples were obtained (18 donors had two antibodies each and three donors each had three antibodies), representing a good range of specificities (Figure 1a). The distribution of antibodies tested in our study is representative of a similar population (Figure 1b). A higher representation of anti-D samples in our set is seen due to the RhD immunoprophylaxis programme where we have received samples from donors to the Australian Red Cross Lifeblood who are deliberately donating their Anti-D-rich plasma.

Sample preparation

Whole blood samples (stored in EDTA anticoagulant solution) containing pre-identified alloantibodies were provided by the Australian Red Cross Lifeblood following a comprehensive ethical approval procedure. After a settling period, 450 μL aliquots of supernatant plasma, containing the antibodies, were dispensed into Eppendorf tubes and frozen at −18°C until needed.

All antibody tests were performed using the $STARGEL_{10}$ system (Haemokinesis, Australia) including anti-human globulin (AHG) gel cards, centrifuge and gel card reader (for recording images of the result). All reagents and plasma samples were equilibrated at 23°C in a temperature and humidity controlled laboratory (50% RH) before testing. Plasma samples were centrifuged for 5 min at 3000 rpm before testing.

Using gel card technology, antibodies were detected via agglutination with antigen-positive reagent red blood cells ($STARGEL_{10}$ 3 cell screen or 11 cell panel). Cells were diluted to 0.8% v/v with a low ionic strength solution (LISS) (Star solution, Haemokinesis, Australia). All cells had double-dose antigen expression except for K, Kp^a, C^w and C. To test plasmas with multiple antibodies, cells expressing only one antigen to the antibodies were present in the mixture, ensuring each antibody was tested individually. Negative control tests against antigen-negative reagent RBCs were also performed.

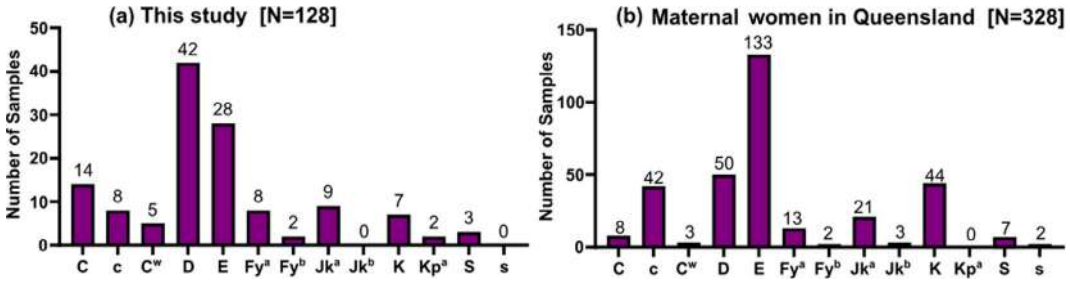

FIGURE 1 Distribution of alloantibody specificities and frequencies. (a) This study's ($N = 128$) donors from Melbourne, Australia. (b) Maternal women in Queensland, Australia ($N = 328$) [15]

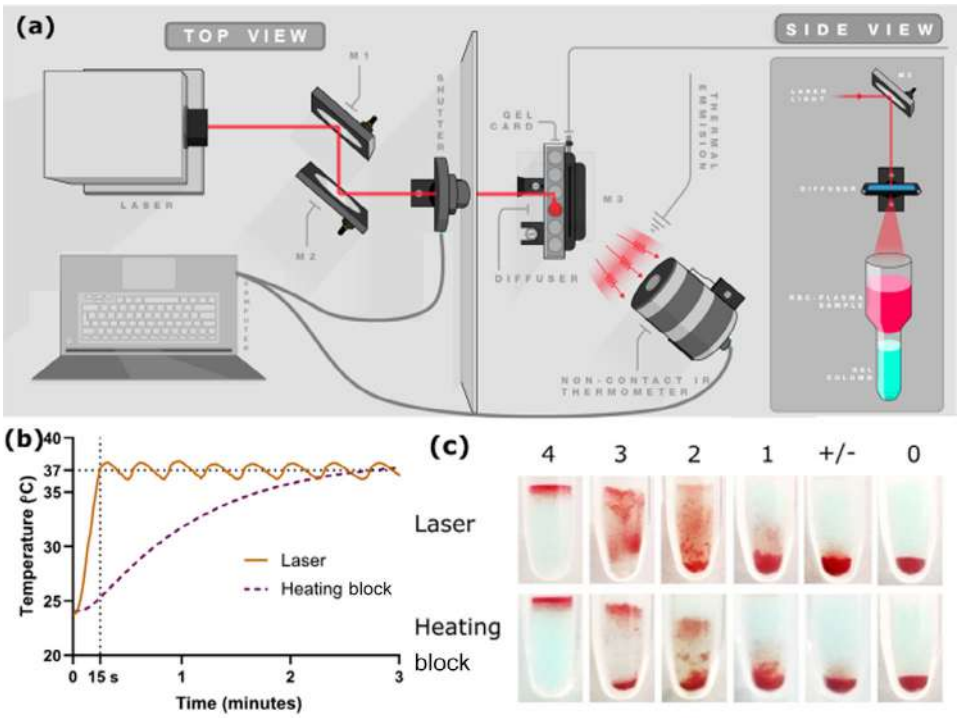

FIGURE 2 (a) Illustration of the experimental set-up. (b) Temperature profiles of plasma–RBC solution in heating block (Haemokinesis, Australia) and laser-based incubation. (c) Grading chart

To conduct an antibody test, 50 μL of prepared cells were added to the gel card upper chamber, followed by 25 μL of plasma. The sample was then heated to 37°C, centrifuged and the agglutination strength result was graded. Comparison tests between heating block and laser were performed on the same day with the same plasma and RBC samples, and graded by the same technician.

Laser incubation

To heat the samples to 37°C, two incubation methods were compared: heating block and laser incubation chamber. Tests performed in the STARGEL$_{10}$ heating block (Haemokinesis, Australia) were incubated for 5 min, following the manufacturer's recommendations, where the sample takes up to 3 min to reach 37°C (Figure 2b).

The laser incubation apparatus illustrated in Figure 2a (Photograph in Section S1) uses an infrared (980 nm) continuous wave diode laser (Dragon Lasers, ChangChun, JiLin, China). A similar experimental set-up to that of Manderson et al. was used [6]. Laser light is directed via three mirrors and an optical diffuser (3 M Scotch Magic Tape 810D over a glass disc) into the gel card from above, illuminating the RBC-plasma sample. The final optical power density on the sample is 4.6 W/cm^2, which consistently heats samples to 37°C in 15 s. A non-contact infrared thermometer detects the thermal emissions from the heated column of the gel card to measure the temperature of the sample. A computer-controlled feedback system monitors and controls the sample temperature by toggling the laser shutter open and close, maintaining the sample temperature between 36°C and 38°C (Figure 2b).

Samples were initially heated for 1 min in the laser incubator. After each incubation, samples were centrifuged and graded. Samples for

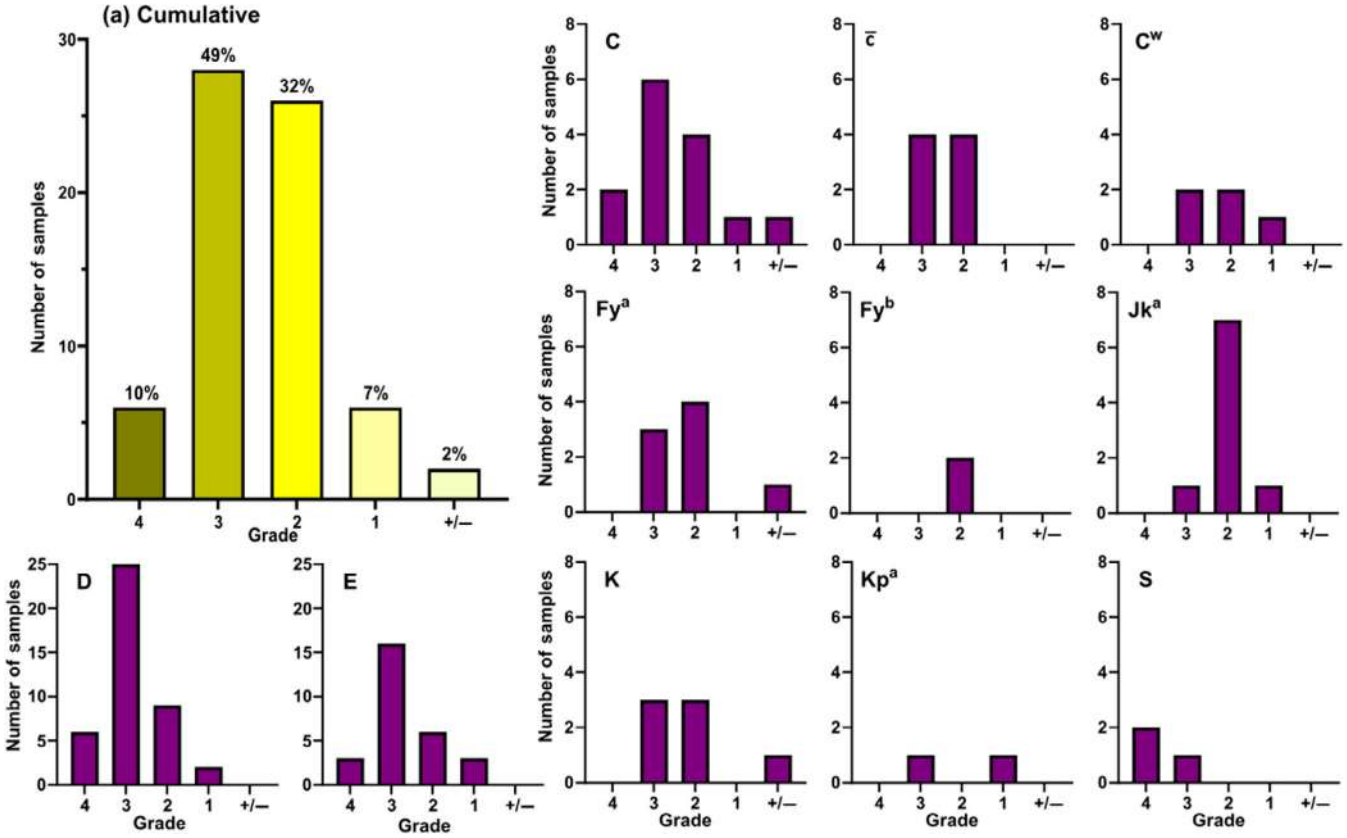

FIGURE 3 Distribution of antibody strengths. (a) Cumulative distribution of agglutination strength across full sample set ($N = 128$) with the heating block method (5 mins); and specific distributions as labelled (C, c, C^w, D, E, Fy^a, Fy^b, Jk^a, K, Kp^a and S)

which the agglutination grade following laser incubation did not match the agglutination grade following the standard heating block incubation were then re-tested via laser incubation in 1-min increments up to 5 min, until the laser test grade matched the grade obtained for the same sample after 5 min with heating block incubation.

Grading

The extent of agglutination, which indicates the strength of the result, may be due to more antibodies being present, the antibody having a higher binding affinity to the antigen, or the RBC having more antigens [14]. By means of antibody–antigen complexes across many cells, large agglutinates of RBCs are formed, which are filtered from the solution through the gel column via centrifugation. Grading refers to the manual assignment of a numerical value to the degree of agglutination observed with values between 0 (negative), where no agglutinates have formed and individual RBCs pass to the bottom of the gel column, and 4 (strong), where strong large agglutinates are formed, which do not pass through the gel column (Figure 2c). A grade of +/− indicates that the sample cannot be definitively graded as 0, but the result is not strong enough to be graded as 1.

Dependent on incubation method, the distribution of RBC agglutinates and unagglutinated cells through the gel cards after centrifugation was consistently different (Figure 2c). Samples incubated in the heating block appear to have a separation of cells in the gel column indicating while some RBCs are agglutinating, other RBCs remain unagglutinated. This difference is especially apparent for samples attributed a grade of 3, where the heating block incubation result has a significant split of cells between the top (agglutinated) and bottom (unagglutinated) of the column. However, samples heated in the laser, which were given a grade of 3, show no unagglutinated cells. This suggests a more uniform level of RBC agglutination is achieved with laser incubation. Despite there being fewer cells concentrated at the very top of the gel column when the sample is incubated by the laser method, there may be more agglutinates overall than that achieved for the same sample incubated in the heating block.

RESULTS

Extent of agglutination across specificities

Across our sample set, a range of strengths of result was seen, as shown by the heating block results presented in Figure 3a. A small number of samples (10%) were very strong, grading a 4 in the heating block test. Most samples (81%) were of moderate strength,

TABLE 1 Example data set showing comparative laser and heating block incubation results

Sample	HB grade (5 min)	Grade after being in laser for			No. mins in laser for:	
		1 min	2 min	3 min	Detection	Equivalency
1	4	4			1	1
2	3	3			1	1
3	3	2	3		1	2
4	2	3			1	1
5	2	1	1	2	1	3
6	1	0	1		2	2
7	+/−	0	0	+/−	3	3

Note: Laser incubation times for detection and equivalent grades as compared to heating block. Samples were incubated in the heating block for 5 min and the grade was noted (yellow, first column). A sample from the same donor was then incubated in the laser chamber. The incubation time required for (a) antibody detection (blue) and (b) to reach the equivalent grade to the heating block test for the same sample (orange) was recorded.

grading either a 2 or 3 in the heating block. Nine percent of samples were weak or very weak, grading either a 1 or a +/−, which is barely distinguishable from a negative. Importantly, a range of strengths was seen for all antibody specificities where the sample size was greater than 2 (see Figure 3, antibody-specific distributions). No single antibody specificity presented as consistently strong or weak.

Incubation by laser

Each sample was incubated in the heating block for 5 min and by laser for the number of minutes required to match the grade achieved by heating block incubation. Two comparisons were made: the length of time required for the laser incubation method to result in a positive antibody detection (grading any strength above 0) and the length of time required for the laser incubation method grade to match that of the heating block. Table 1 presents an example subset of the comparative incubation data analysis showing antibodies of seven plasma samples. Laser incubation grades coloured blue indicate that the antibody was detected after the indicated duration of laser incubation, but the result was weaker than that of the 5-min heating block incubation. In all cases of a lower grade with laser incubation, the same sample was then re-tested with a longer laser incubation. Laser incubation grades coloured orange match or exceed those of the heating block incubation for that sample, after which no longer duration laser incubation testing of that sample was conducted.

Figure 4 presents the effect of laser incubation time on antibody detection and grade equivalency with the standard heating block incubation method for all samples ($n = 128$). These results indicate that for 96.1% of samples (123/128 measured), the presence of the antibody can be correctly detected after only 1 min of incubation in the laser (Figure 4a). These 123 antibodies represent a range of strengths including several that are very weak (Figure 4b). Only five samples, which all showed very weak agglutination using the standard 5 min of heating block incubation, took longer than 1 min to be detected via laser incubation. No samples required longer than 3 min of laser incubation in order to detect the antibody. That is, in all instances, antibody detection was faster using laser incubation than the standard heating block method.

All samples were also tested with antigen-negative reagent RBCs. After 5 min of incubation by laser and separately also by heating block for comparison, all except one (127) produced the expected negative (grade 0) result. The one that was not negative was known to host an anti-S antibody (grade 4) and gave a grade of 2 against all other cells expected to be negative. It is most likely that this plasma contains an antibody that had not been previously identified and to which we do not have a negative cell. More work is required to identify this antibody and test against a true negative cell. The same result was seen for both the laser and heating block, indicating that this result was not an artefact of laser incubation.

Further, after a 1-min laser incubation, 86 samples (67.2%) achieved a result of equivalent strength to that of a 5-min heating block incubation. Forty-two took longer than 1 min of laser incubation to achieve equivalency (Figure 4c). When the antibody is very strong, (heating block incubation grade 4), it is very likely to be detected with the equivalent strength using a laser incubation of 1 min. When the antibody is weaker (grading 3, 2, 1 or +/−), it can take up to 5 min of laser incubation to achieve a grade equivalent to that of the 5-min heating block incubation. For the three samples that required 4 min of laser incubation for equivalency, two of these were easily detected (grading 2) in 1 min and only required 4 min to bring the grades up to 3 to match the heating block result for the same sample. One sample required 4 min to bring it up from a barely detected +/− to a clearer positive graded of 1. This result indicates that this sample hosted a particularly weakly agglutinating antibody. One sample required 5 min of incubation by laser for an equivalent grade to that of heating block incubation. This sample was detected after only 2 min of laser incubation, but took a further 3 min to bring that grade up to a grade of 2, to match that of the heating block. For all samples, incubation by laser gave the same or stronger result within 5 min. No samples required longer than 5 min to achieve an equivalent result to that of the 5-min heating block incubation.

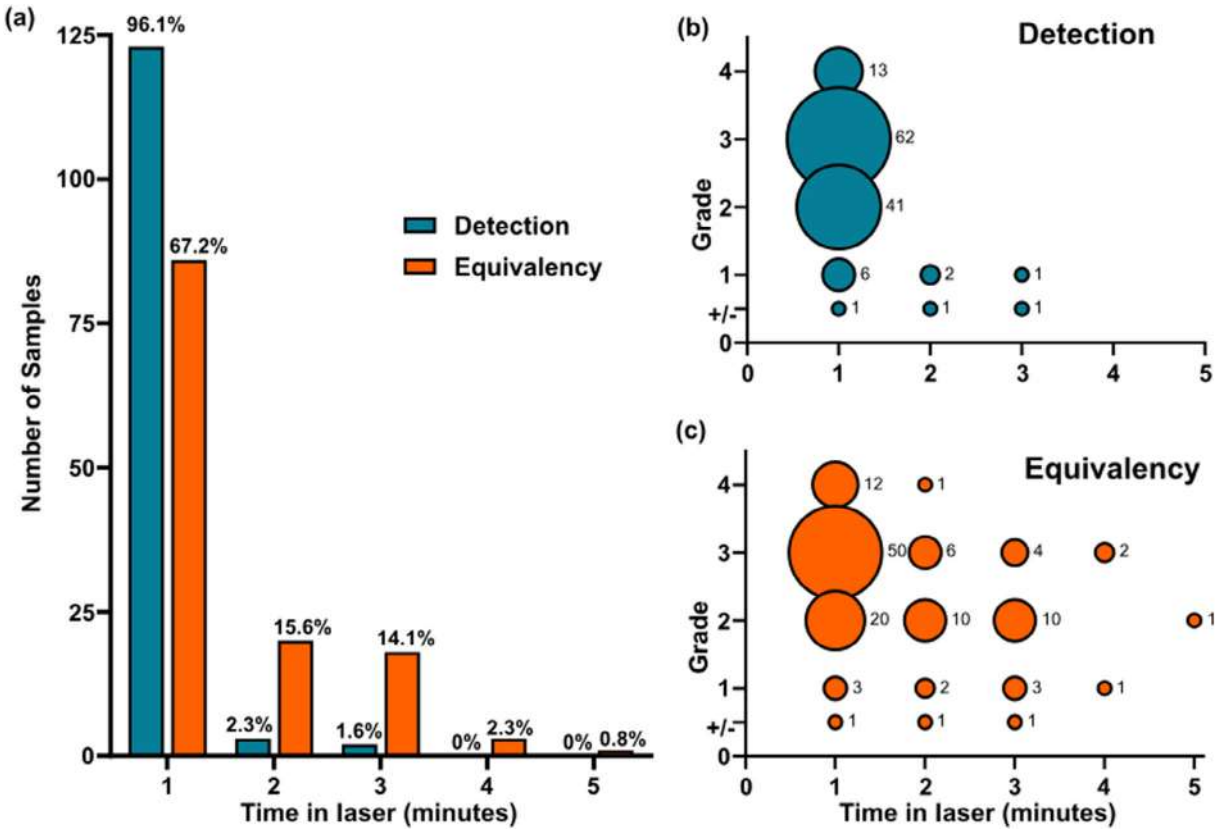

FIGURE 4 Incubation time in laser chamber. Number of minutes of incubation by laser for any detection of antibody (blue) and length of time to achieve an equivalent result (orange) to that of a 5-min heating block incubation. (a) Cumulative detection and equivalency for all samples; size of bubbles represents the number of samples for (b) incubation time for detection of antibodies of different sample strengths, and (c) incubation time required by laser to achieve equivalent strength results

DISCUSSION

Little research into immunohaematological incubation methods and outcomes has been carried out since the IAT's inception in the 1940s [2–6]. Here, with laser incubation technology, the IAT is being brought into the 21st century. Laser incubation occurs via a photothermal energy conversion mechanism. Laser light (energy) can penetrate deep into the sample, where it is absorbed and converted to heat directly within the sample itself. By contrast, heating block incubation is based on thermal conduction. Heat is generated by an electrical element and must be conducted slowly and inefficiently through the air gap around the gel card, then through the gel card material (polypropylene), before heating the sample from the outside-in. As polypropylene and air are both insulators, heating by conduction is inherently slow. This is further aggravated by the heating block temperature being fixed at 37°C to avoid overheating. Raising the sample temperature to the required 37°C therefore leads to a longer required total time using heating block incubation to achieve a correct antibody test result.

Our system (Figure 2a) uses a 980 nm laser as the energy source for incubation of gel cards. Because RBCs preferentially absorb infrared light, this specific selection of wavelength means the surface of the RBC itself, where the antigens that bind with the antibodies are located, is preferentially heated [6]. Energy and therefore heat are thus rapidly and directly provided by laser incubation at the reaction site itself, the locus for the antigen–antibody complexation, rather than waiting for conduction through the bulk fluid surrounding the reactants. Indeed, the surface temperature of the RBCs themselves may be higher than the bulk fluid temperature during laser incubation, which may further lead to the observed enhanced reaction rate.

The differences in directness and efficiency of the heating mechanisms (photothermal using laser vs conduction using heating block) are certainly critical to the measured improvement in the method when using laser incubation. However, there may also be other contributing factors that warrant further investigation. For instance, the kinetics and thermodynamics of the antibody–antigen complexation reaction, as well as the possibility that rapid heating induces reconfiguration or relaxation of the antibody macromolecule in solution, render the locus of complexation more accessible by reducing steric hindrance or increasing flexibility of the active site.

Our analysis of 104 donor samples representing 128 alloantibodies demonstrates that laser incubation is a viable and robust method of incubating plasma–RBC samples for detection of red cell alloantibodies. Laser incubation can rapidly detect antibodies and can produce comparable results to those of the standard method in faster timeframes. The enhanced localized heating described causes rapid

binding of antibody to antigen—giving correct positive results within 1 min for 96% of samples (Figure 4a).

This laser incubation method correctly detects antibody specificity. Where pre-identified antibodies were confirmed through our testing using positive and negative antigen-specific RBCs. Further, for one sample, we detected an antibody that had previously not been identified by the Australian Red Cross Lifeblood, who provided us with all samples. Laser incubation can be used reliably for antibody detection and also for identification of specificity as well.

We have also shown that the time it takes for a positive result to be detected is independent of antibody specificity. Each antibody tested showed a range of strengths among our representative population of donors, and the grade distribution appears to be consistent regardless of group or antibody (Figure 3). As our study used donor samples, there were some groups and antibodies for which an insufficient number of samples were available to clearly demonstrate this distribution. Further testing is therefore needed to confirm this result for some groups and antibodies.

In addition to providing specifically correct results in a shorter amount of time than heating block incubation for all samples, laser incubation is non-destructive for clinical samples (See Section S2 and S3). The IAT was conducted using the standard 5-min heating block incubation using plasma samples and RBC suspensions that had previously and separately been subjected to laser incubation. The laser incubated samples showed identical binding performances to non-laser incubated samples, indicating that laser incubation does not damage or affect the binding affinity of either the antibodies or RBC antigens.

Beyond the improvements demonstrated in this work with regards to immunohaematology and the IAT, the advantages of laser incubation can be applied to the heating of blood and plasma samples, or indeed any antibody–antigen colloidal suspension, for a range of pathology and antibody tests that require incubation above room temperature, or controlled thermocycling.

In conclusion, we have applied laser incubation technology to rapidly detect the antibodies in the plasma of over 120 antibody-positive donors via the IAT in gel cards. Representing several key blood group systems, we have tested 11 clinically significant antibodies and found all to present a range of agglutination strengths. Laser incubation can rapidly detect even very weak antibodies using CAT/gel and the laser does not damage antibodies or RBCs. We have successfully detected the presence of an antibody—critical to prevent life-threatening haemolytic reactions—after only 1 min of laser incubation for 96% of samples. In all instances, antibody detection was faster using laser incubation than the standard heating block method. In comparison with traditional heating block incubation, it is clear that the difference in heat generation and heat transfer mechanisms between the laser and heating block plays a critical role in the reduction in reaction time required to generate a correct result using laser incubation. We demonstrate that the infrared laser technology is a safe, reliable and rapid form of incubation for blood samples. Use of laser incubation technology for blood sample analysis and antibody screening can thus lead to improvements in standard testing, particularly with regard to testing times, which can translate directly to improved patient outcomes.

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All authors (except D.A.) contributed to the design of the research project. C.A.M. designed and built the laser incubation chamber, analysed the data and wrote the first draft of the manuscript. H.M. performed the experiments and graded the results. D.A. performed the flow cytometry experiments and analysis. J.M. contributed essential reagents and equipment. J.T. and G.G. supervised the study. All authors reviewed the manuscript. Australian Research Council Linkage Project LP110200973. Financial and in-kind contributions from Haemokinesis Pty Ltd, including reagent red cells, gel cards, LISS, centrifuge. Australian Red Cross Lifeblood for donor clinical blood samples.

CONFLICT OF INTEREST

J.M. is the CEO of Haemokinesis Pty Ltd. The other authors declare no conflict of interest.

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SUPPORTING INFORMATION

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

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ORIGINAL ARTICLE

Vox Sanguinis ISBT International Society of Blood Transfusion

Molecular biology analysis of ABO blood group variants caused by natural chimaerism

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Abstract

Background and Objectives: The chimaerism phenomenon constitutes a significant mechanism underlying ABO phenotype discrepancies; however, its detection has technical challenges. In the current study, we explored different techniques to establish the chimaeric status of ABO blood types.

Materials and Methods: Fifteen individuals with possible chimaeric ABO blood type, as suggested by standard tube or column agglutination method and RBC adsorption-elution test, were enrolled in the study. The red blood cells from 11 investigated subjects showed mix-field agglutination with anti-A or anti-B in blood typing; weak A or B antigens on the other four individuals' RBCs were detected by adsorption-elution tests. The genetic study was conducted with PCR-SSP genotype, DNA sequencing of the ABO gene, STR analysis and ddPCR.

Results: The genetic chimaeric status was confirmed in four (27%) individuals by SSP test alone. The ABO gene sequencing identified an additional ABO allele and enabled chimaerism detection in 10 (67%) subjects. The STR analyses established the chimaerism status in 13 (87%) individuals. In the two cases where neither of the tests mentioned above had positive findings, the ddPCR was adopted, and microchimaerism, with an extremely low degree of chimaerism (0.77% and 0.12%), was revealed. The ddPCR revealed the unequal haplotypes (29.5% *B* vs. 70.5% *O*) in one subject and distinguished this *B/O-O/O chimaera* from certain B subgroups (*B/O* genotype without any mutation) like B_3.

Conclusion: The ABO blood type chimaerism can be genetically established by comprehensive molecular methods, including PCR-SSP/DNA sequencing, STR and ddPCR, which is particularly sensitive for the detection of microchimaerism.

Keywords

ABO blood grouping discrepancies, blood groups, genotyping, natural blood chimaerism, transfusion strategy

Highlights

- The detection rate of natural blood group chimaeras could be greatly increased by gradually adopting PCR-sequence-specific priming/DNA sequencing, short-tandem-repeat analysis and digital droplet (dd) PCR.

Yuqing Wang and Qiuju Mou contributed equally to this study.

- ddPCR is also effective for microchimaeras, which have not been identified by many current laboratory methods.

INTRODUCTION

A chimaera is characterized as an organism that contains cells from two or more separate zygotes [1], and the human chimaera phenomenon was first revealed in blood group typing studies in 1953 [2]. Presence of chimaeric red blood cells poses special difficulties in the identification of the ABO blood group, and transfusion related to blood group typing chimaeric individuals may be complicated with hemolytic reactions [3]. Latest studies have suggested that natural chimaeras might be much more common than originally presumed in the general population [1, 4]; however, identification of chimaerism in the clinical transfusion laboratory is still challenging.

Serologic tests are still the most commonly used method to screen for blood group-type chimaeras. Individuals with chimaeric red cells usually show ABO blood group discrepancy in blood typing studies, presenting mixed-field agglutination or other ABO typing abnormalities. However, serological studies alone cannot establish the status of chimaerism and further genetic evidence is mandatory to prove the existence of two sets of genomes in an individual.

In the current study, we explored using the polymerase chain reaction (PCR)-SSP (sequence-specific priming) genotype and DNA sequencing, short tandem repeat (STR) analysis and digital droplet PCR (ddPCR) to establish the chimaeric status of ABO blood types suggested by serological studies.

MATERIALS AND METHODS

Samples

A total of 15 individuals with suspected chimaeric ABO blood type suggested by standard tube or column agglutination method and RBC adsorption-elution test were enrolled in the study. The red blood cells from 11 investigated subjects showed mix-field agglutination with anti-A or anti-B in blood typing; weak A or B antigens on the other four individuals' RBCs were detected by adsorption-elution tests. The patients were without identifiable risk factors for acquired variant ABO phenotypes, such as ABO-incompatible transfusion, transplantation, hematologic disorders, infection and cancer diseases (Table 1).

Informed consent was obtained, and the study was approved by both the ethics committee of the Shanghai Blood Center and the ethics committee of Ruijin Hospital, Shanghai Jiao Tong University School of Medicine.

ABO gene PCR-SSP

The genomic DNA was isolated from whole blood using a DNA isolation kit (QIAquick, Qiagen GmbH, Hilde, Germany). The PCR-SSP assay kit (Tianjin Super Biotechnology Development Co., Ltd, Tianjin, China) for ABO genotyping, which targeted informative SNVs at the ABO gene locus, was adopted in the study. The presence of each allele of certain designated loci was picked up by specific binding between the DNA segment and PCR primer with 3′ complement sequence. Multiple PCR amplifications targeting different loci were performed in Table S1, and the yield of the PCR was visualized after electrophoresis in 2% agarose gel.

ABO gene sequencing

Bidirectional sequencing of the enhancer, promoter, all exons, and flanking intronic region of ABO gene was conducted with genomic DNA samples. The primers sequences and amplification conditions for the polymerase chain reaction were described previously [5, 6]. The exon 7 of the ABO gene, encoding around 70% of ABO cDNA and containing most ABO allele-specific sites identified so far, is most informative to determine the haplotypes of a specific individual and was chosen for chimaerism analysis in the study. If necessary, the gel-purified PCR products of exon 7 were cloned into the pMD18-T vector (TaKaRa, Dalian, China) and sequenced to confirm the type and amounts of haplotypes.

STR analysis

Fifteen STR loci (CSF1PO, D13S317, D16S539, D18S51, D19S433, D21S11, D2S1338, D3S1358, D5S818, D7S820, D8S1179, FGA, TH01, TPOX, and VWA) and the amelogenin locus were amplified using a commercial kit (Applied Biosystems, Woolston, Warrington, UK). The STR loci were adopted due to high polymorphism, genetic stability, and low mutation rate among the Chinese population. The experiment was carried out according to the manufacturer's instructions. Briefly, the concentration of genomic DNA isolated from whole peripheral blood was measured with NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). The PCR was conducted on ProFlex Base PCR thermal cycler (Applied Biosystems, Singapore) in a total volume of 10 μl, containing 1 ng of genomic DNA. The thermal profile included 11 min of initial denaturation at 94°C, 28 cycles of amplification (1 min at 94°C, 1 min at 59°C, 1 min at 72°C) and 60 min of final elongation at 60°C. Prior to separating the PCR fragments by capillary electrophoresis, 1 μl amplification products was added to 8.5 μl of deionized formamide Hi-Di™ (Applied Biosystems) and 0.5 μl GeneScan™ 600 LIZ® Size Standard (Applied Biosystems) for STR analysis. Amplification products were further separated on an ABI PRISM 3130xl Genetic Analyzer. The raw data were analysed using GeneMapper ID v3.2 software (Applied

TABLE 1 The ABO blood group chimaeras identified by SSP genotype, DNA sequencing, short tandem repeat analysis and droplet digital PCR

No.	Sample	Suspected ABO chimaeric antigen in normal antigen	ABO genotyping by SSP	Detected alleles and additional allele by sequencing	Informative STR loci (*n*)[a]	ddPCR numbers of FAM-positive droplets/ (FAM + VIC-positive droplets)
1	S50	A in O	*A1/O2*	***O2/O2 and A1***	**2**	-
1.1	S50's father	A	-	*A1/O2*	-	-
1.2	S50's mother	B	-	*B1/O2*	-	-
2	S92	B in A	*A1/B*	***A1/O1 and B***	**1**	-
3	S114	B in O	*O2/O2*	*O2/O2*	0	**5/648 (0.77%)**
4	S129	A in B	*A1/B*	***A1/B and O1***	**1**	-
5	S158	B in A	*A1/O1*	*A1/O1*	0	**5/4097 (0.12%)**
6	S163	B in A	*A1/B*	***A1/A1 and B***	**2**	-
7	S519	B in O	***O1/O2/B***	***O1/O2 and B***	**3**	-
8	S583	A in O and B in O	***A1/B/O1/O2***	***A1/B and O2***	**6**	-
9	S720	B in A	***B/O1***	***A1/O1 and B***	**4**	-
10	S739	B in O	*B/O1*	*B/O1*	**1**	**958/3247 (29.5%)**
11	S756	A in B	*B/O2*	***B/O2 and A1***	**4**	-
12	S835	B in A	***A1/O1/B***	***A1/O1 and B***	**3**	-
13	R97	B in A	*B/O2*	***A1/O2 and B***	**3**	-
14	R163	A in O and B in O	*A1/B*	*A1/B*	**8**	-
15	R188	B in O	*O1/O1*	*O1/O1*	**1**	**104/1961 (5.30%)**
15.1	R188's mother	B	-	*B/O1*	-	-
15.2	R188's father	B	-	*B/O1*	-	-
	Neg Ctrl[b]	A,O	-	*A1/O1/O2*	-	0/2917
	Norm Ctrl[c]	B	-	*B/O1*	-	493/942 (52.3%)
	Detective rate		27% (4/15)	67% (10/15)	87% (13/15)	-

Note: The ABO alleles are presented in the format listed in Table S1. The positive findings regarding the status of chimaerism by each method are highlight in bold. Limited by the length of the table, we expressed *ABO*O.01.01* as *O1*, *ABO*O.01.02* as *O2*. -, not tested.

[a]'*n*' indicates the numbers of STR loci with positive findings, which present either three peaks or abnormal low peak.

[b]Neg Ctrl: Mixture of blood donors' gDNAs containing *A1,O.01.01* and *O.01.02* was set as negative control for identifying B chimaera.

[c]Norm Ctrl: B blood donor's gDNA with *B.01/O.01.01* genotype was set as normal *B/O* diplotype control.

Biosystems), with a minimum interpretation peak threshold of 50 relative fluorescence units (RFU).

Droplet digital PCR

Target genomic loci of *ABO*B* in four samples, S114, S158, S739 and R188, were quantified by ddPCR with QX200 (BIO-RAD, Hercules, California, USA) based on approximately 12,000–15,000 nanolitre-sized droplet reactions.

The *ABO*B.01* allele is distinguished from *A1* and *O.01* mainly at six single nucleotide substitutions at base position c.526, c.657, c.703, c.796, c.803 and c.930; however, several rare ABO blood group variants such as cisAB or B(A) may carry *B*-specific SNPs at positions c.526, c.703, c.796, and c.803 [6]. Therefore, target-specific probes were focused on *ABO* polymorphism c.657T (*rs*8176741), which was *B* specific and also not included in rare ABO variants. Primers and probes were designed by Primer Premier 5.0. The primers were ABO-657CT-F 5′-GTGGATTACCTGGTGTGCGT-3′ and ABO-657CT-R 5′-TGCAGGGTGCCGAACA-3′. ABO gene SNP c.657C base was detected by the ABO-657CT-P-C VIC-5′-CCCACGTGGTCGC-3′ probe, while the c.657T base was detected by the ABO-657CT-P-T FAM-5′-CCCACATGGTCGC-3′ probe (n.657 site was underlined).

The limit of the blank (LOB) was set as the highest mutant (MT, *B.01* allele) copy number concentration that might be found when replicates of a blank (=devoid of MT copies) are tested: $LOB = Mean_{copy\ number\ sample} + 1.645 \times SD_{copy\ number\ blank}$ [7]. The limit of detection (LOD) was set as the lowest copy number concentration that could be distinguished from the LOB with >95% certainty: $LOD = LOB + 1.645 \times SD_{low\ mutant\ copy\ number\ sample}$ [7]. However, due to the absence of an MT standard, MT DNA from sequencing known human blood sample was serially diluted in a background of sequencing known human wild type (WT, *O.01* allele) DNA to determine the linearity. A total of six successive gradient dilutions (1:10, 1:100, 1:1000, 1:2000, 1:5000, 1:10,000), that is a MT/WT DNAs ratios 0.10000, 0.01000, 0.00100, 0.00050, 0.00020, 0.00010, were

done and detected using a dual probe mixture test. For determining the limit of quantification (LOQ) of ddPCR, analysis was carried out at a series of MT dilutions ((MT/WT DNAs ratio) 0.10000, 0.01000, 0.00500, 0.00100, 0.00050, 0.00025, 0.000125, 0.0000625), and three tests were conducted under the same reaction conditions to calculate the CV of the method and evaluate the repeatability of the method.

Fifteen normal B blood group gDNA samples heterozygous for *B.01* allele (MT) and five normal O blood group gDNA samples homozygous for *O.01* allele (WT) by DNA sequencing were diluted to 1 ng/μl to determine the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), negative likelihood ratio (NLR) and accuracy for ddPCR. The diagnostic performance of ddPCR was calculated by MEDCALC (https://www.medcalc.org/calc/diagnostic_test.php) [8]. Thermal cycling was performed as follows: step 1, 95°C for 10 min (1 cycle); step 2, 94°C for 30 s followed by 60°C for 1 min (40 cycles); step 3, 98°C for 10 min (1 cycle); and step 4, hold at 4°C. The percentage of *ABO*B.01* chimaerism was calculated according to the following formula: *ABO*B.01* chimaerism (%) = number of droplets of T bases/(number of droplets of T bases + number of droplets of C bases).

Since ABO gene sequencing, SSP and STR all failed to detect chimaerism in subjects S114 and S158, ddPCR was applied to the samples for chimaeric analysis. For subjects S739 and R188, neither ABO gene sequencing nor SSP were informative, and the STR analysis showed dubious results with only one locus out of 15 STR loci, so the ddPCR was also introduced in the chimaeric status examination on these two samples. The status of chimaerism in case S739 could not be confidently differentiated from the *B/O* diplotype of an individual with certain ABO subtypes with STR analysis alone. The ddPCR was then conducted to determine the ratio of *B.01* and *O.01.01* alleles in the subject. A mixture of blood donors' gDNAs containing *A1,O.01.01* and *O.01.02* was set as the negative control, and B blood donor's gDNA with *B.01/O.01.01 genotype was set as* normal *B/O* diplotype control.

Nomenclature of mutations and ABO alleles

The ABO alleles were named according to the nomenclature used by the International Society of Blood Transfusion.

RESULTS

ABO-SSP analysis

According to the PCR-SSP typing table (Table S1), the blood group SSP genotyping showed that there were more than two ABO alleles amplified in four (27%) cases (No. 7, 8, 9, 12 in Table 1), indicating the status of chimaerism.

ABO gene sequencing

No ABO subgroup-specific mutations were found by DNA sequencing in all subjects studied. Additional copies of exon 7 and enhancer were revealed in the sequencing results in 10 (67%) out of 15 individuals, indicating the presence of extra *ABO* alleles and chimaerism (Table 1). The representative sequencing results on exon 7 acquired from sample S92 are presented in Figure 1.

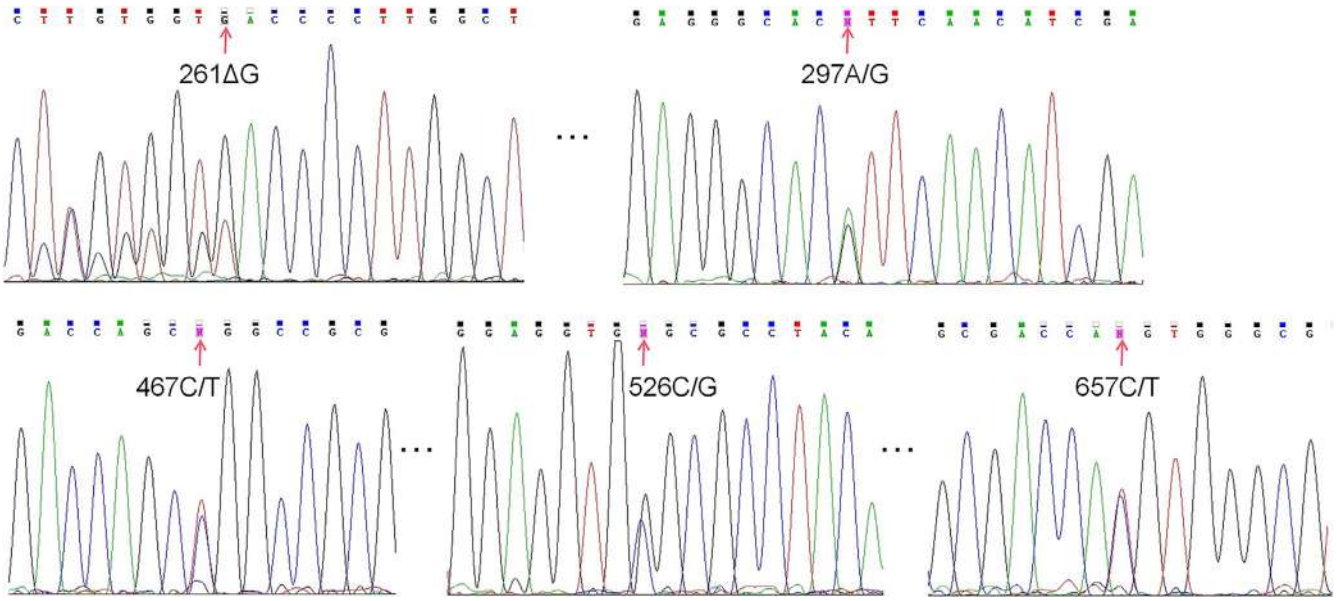

FIGURE 1 DNA sequencing analysis of blood group chimaera. Heterozygous state of c.261G/delG, c.297A/G, c.467C/T, c.526C/G, and c.657C/T mutations in sample S92 (No. 2) indicates the presence of three alleles, *ABO*O.01.01*, *ABO*A1.02*, and *ABO*B.01*, confirming the status of ABO blood type chimaerism of the individual

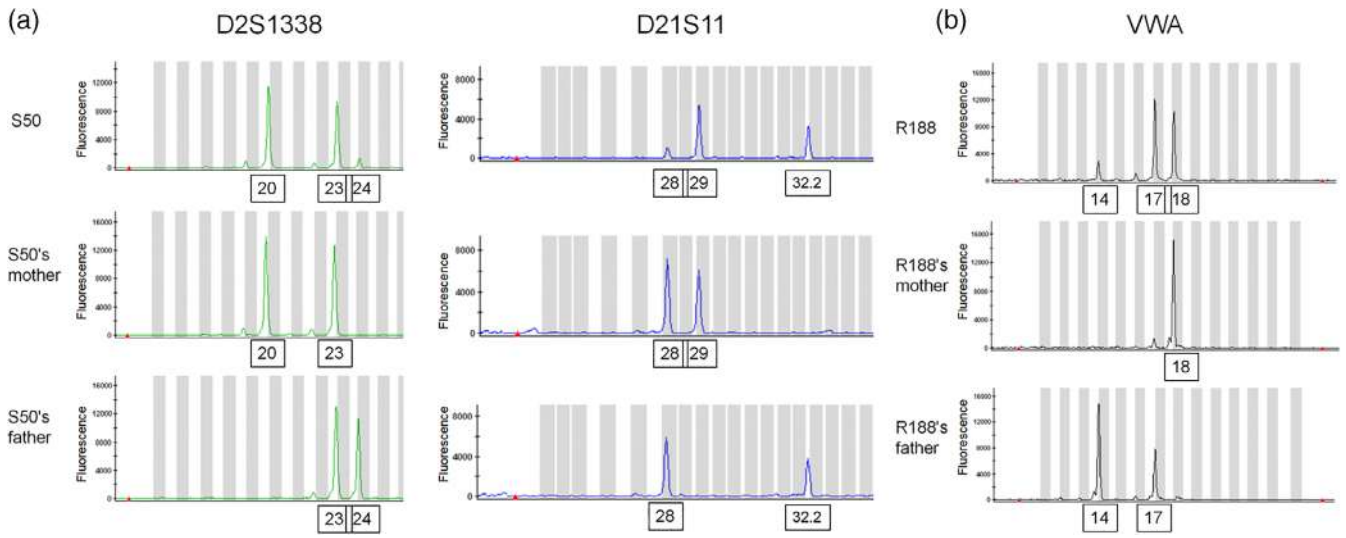

FIGURE 2 Short tandem repeat analysis in the two pedigrees from peripheral blood DNA. (a) Double paternal DNA was detected in D2S1338 and D21S11 in sample S50 (No. 1). (b) Three fragments as 14, 17, and 18 were detected in VWA in sample R188 (No. 15)

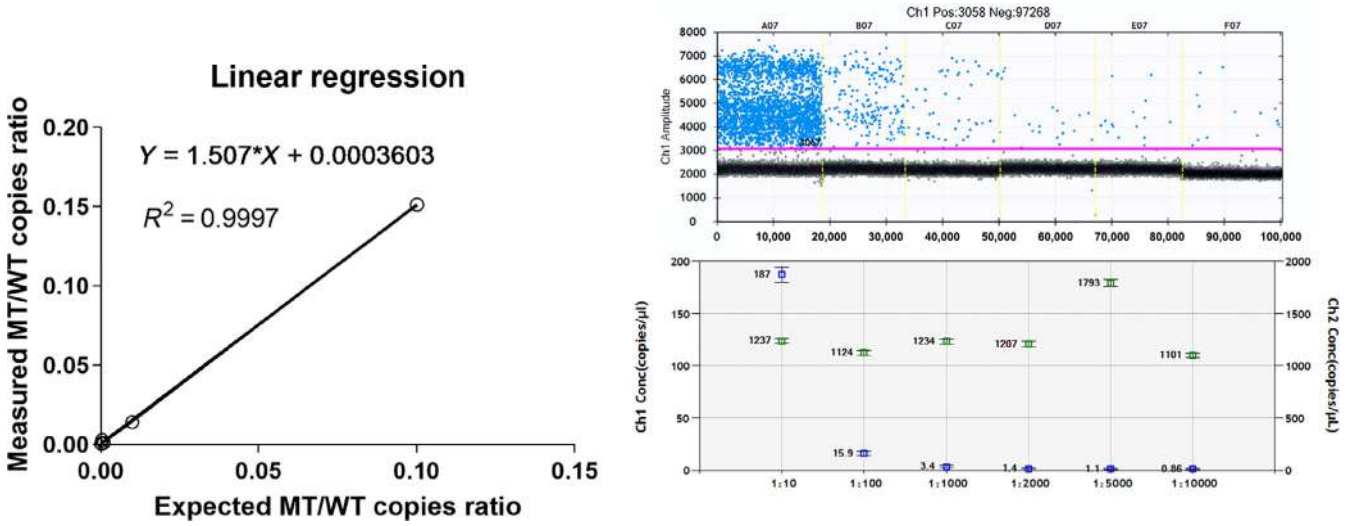

FIGURE 3 Linear regression between ddPCR output ratio and actual DNA ratio. (a) Valuation of linear dynamic range and dilution linearity. The ddPCR was performed on mixtures of MT DNAs and WT DNAs with known ratios (1:10, 1:100, 1:1000, 1:2000, 1:5000, 1:10,000). A linear relationship was observed between the expected ratio of MT DNAs to total DNAs and the actual ratio. (b) Droplet scatter plots of templates with different concentrations were amplified by digital PCR. (c) The ddPCR quantitative gradient dilution droplet concentration

TABLE 2 The repeatability evaluation dilution gradient detected

ddPCR-ABO-C657T							
	Accepted reactions (copies/20 μl well)						
Dilution	N1	N2	N3	*N* mean	Theoretical value	SD	CV
1×10^{-1}	2480	2320	2440	2413.33	4000	83.27	3.45%
1×10^{-2}	296	298	308	300.67	400	6.43	2.14%
5×10^{-3}	68	44	52	54.66	200	12.22	22.35%
1×10^{-3}	28	18	22	22.67	40	5.03	22.21%
5×10^{-4}	5.6	6.2	8	6.6	20	1.25	18.92%
2.5×10^{-4}	5.6	8	4.4	6	10	1.83	30.55%
1.25×10^{-4}	3.2	6.6	3.8	4.53	5	1.81	40.03%
0.625×10^{-4}	No call	No call	No call	-	2.5	-	-

STR analysis

Among all individuals investigated, 13 (87%) of them showed three peaks on at least one of the STR loci examined, establishing the status of chimaerism. Notably, the RBCs of sample S739 presented mixed-field agglutination of B/O and O/O, and ABO gene sequencing revealed no genetic mutations, thus strongly suggestive of a high degree of chimaerism of blood type. The PCR-SSP/ABO sequencing failed to differentiate the high degree of chimaerism and heterozygous status of ABO alleles in this individual and only showed the coexistence of *B* and *O* alleles. However, the STR study enabled us to establish the chimaeric status in the subject and showed that S739 is a chimaera (with only one locus D2S1338, Figure S1).

Probands of two unrelated pedigrees, S50 and R188, were identified as dispermic chimaerism by STR analysis with DNA isolated from the proband and the parents (Figure 2). In these two cases, two paternal genes and one maternal gene were detected by STR analysis in the probands.

The ddPCR analysis

The ddPCR was of high sensitivity and specificity. Ten were no detectable MT copies in any of the LOB pools, giving a LOB <1 copies/μl. However, 3 ABO-C657 positive droplets (1.4 copies/20 μl well, 1.4 copies/20 μl well, and 1.6 copies/20 μl well, respectively) were found in 3 of 10 blank samples. Based on this, we set the LOB 1.61 copies/20 μl well for all assays. When MT dilution concentration was 1.25×10^{-4}, the three samples with low mutation copy numbers were 3.2 copies/20 μl well, 6.6 copies/20 μl well, and 3.8 copies/20 μl well, respectively. The LOD was calculated to be 4.59 copies/20 μl well, according to the formula. All analytical points showed more than 10,000 droplets and were therefore retained for further analysis. The standard curve was $Y = 1.507X + 0.0003603$, and its linear regression coefficient $R^2 = 0.9997$, indicating that the theoretical and actual ratio of MT/WT presented a good linear relationship under such conditions (Figure 3). In order to determine the LOQ of ddPCR, MT samples of 4000 copies/20 μl well, 400 copies/20 μl well, 200 copies/20 μl well, 40 copies/20 μl well, 20 copies/20 μl well, 10 copies/20 μl well, 5 copies/20 μl well and 2.5 copies/20 μl well were tested for three times (Table 2). When the dilution MT sample was 20 copies/20 μl well, the CV value was 18.92%, and the LOQ for the assays was 6.6 ± 1.25 copies/20 μl well. The MT copies of the samples S114, S158, S739 and R188 were higher than this value; therefore, the acceptable threshold of quantitative detection was achieved, and the established ddPCR system worked stably and had good repeatability. The sensitivity, specificity, PPV, NPV, NLR and accuracy of ddPCR were 93.75% (95% CI: 69.77%–99.84%), 100% (95% CI: 39.76%–100.00%), 100%, 80% (95% CI:37.48%–96.39%), 0.6 (95% CI: 0.01–0.42) and 47% (95% CI: 75.13%–99.87%), respectively (Table 3).

The ddPCR analysis of samples S114 and S158, which had negative findings in all the other methods, confirmed the presence of

TABLE 3 Diagnostic performance of ddPCR in this study

	Clinically MT positive	Clinically MT negative	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)	Negative likelihood ratio (95% CI)	Accuracy (95% CI)
ddPCR								
Test positive	15[a]	1[b]	93.75% (69.77%–99.84%)	100% (39.76%–100.00%)	100% (NA)	80.00% (37.48%–96.39%)	0.06 (0.01–0.42)	95.00% (75.13%–99.87%)
Test negative	0[c]	4[d]						

Note: Sensitivity = [a/(a + c)] × 100%; Specificity = [d/(b + d)] × 100%; PPV = [a/(a + b)] × 100%, positive predictive value; NPV = [d/(c + d)] × 100%, negative predictive value; Negative likelihood ratio = (1 − Sensitivity)/Specificity; Accuracy = [(a + d)/(a + b + c + d)] × 100%; Calculated by MEDCALC (https://www.medcalc.org/calc/diagnostic_test.php).
Abbreviation: NA, not applicable.

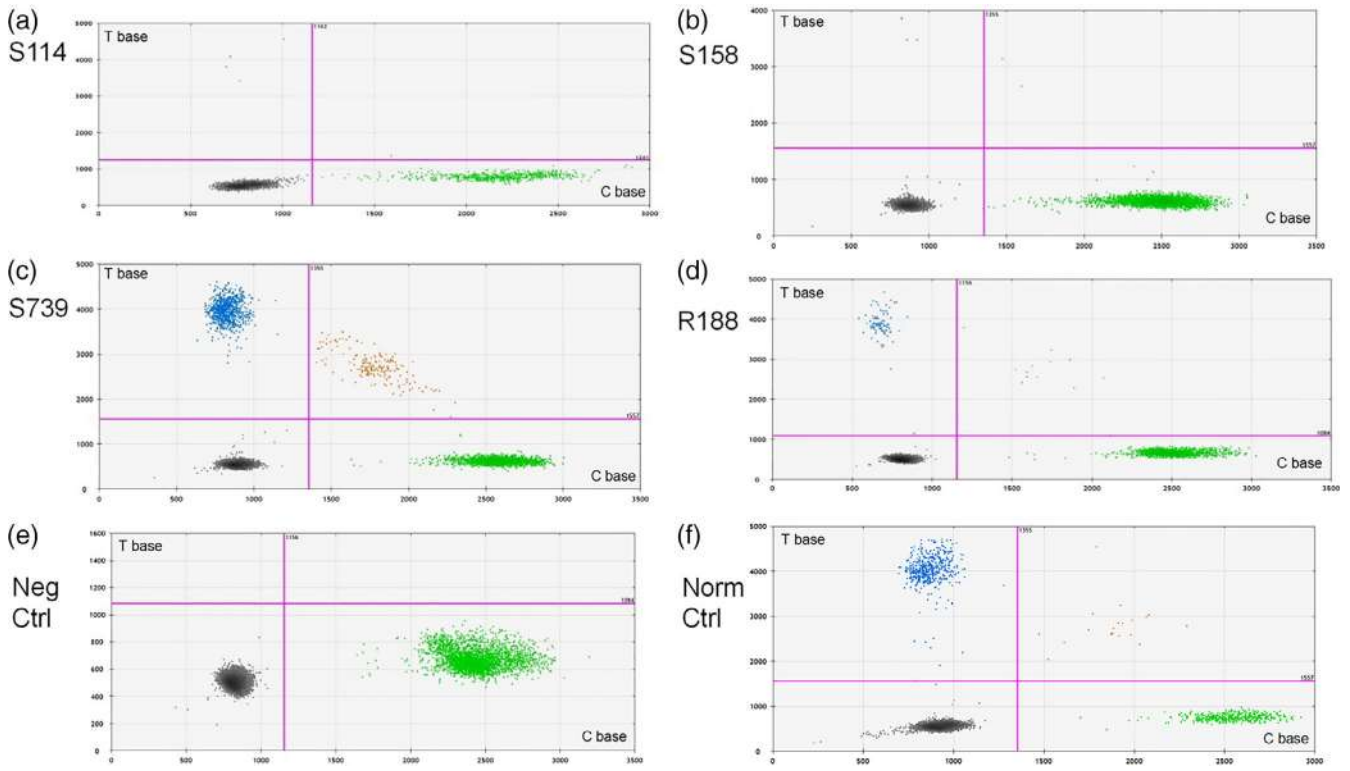

FIGURE 4 Droplet digital PCR analysis of blood group microchimaera. Channel 1 amplitude in FAM with T base specific primer and channel 2 amplitude in VIC with C base specific primer. The results demonstrated that *ABO*B.01* was present in samples S114 (a) and S158 (b) at extremely low ratios 0.77% (20 copies/20 μl well) and 0.12% (10.2 copies/20 μl well), respectively. 29.5% *B.01* (1880 copies/20 μl) was detected in sample S739 (c) and 5.30% in R188 (174 copies/20 μl well) (d). Negative control and normal control were shown in (e) and (f), respectively. In normal control, 52.3% *B* (1042 copies/20 μl) and 47.7% *O* (948 copies/20 μl) were determined

microchimaerism (chimaerism ratio < 1%) (Figure 4a,b). The percentage of *ABO*B.01* microchimaerism in samples S114 and S158 was ultimately 0.77% (20 copies/20 μl well) and 0.12% (10.2 copies/20 μl well), respectively, as calculated with the *ABO*B.01* chimaerism formula (Table 1). By ddPCR, the chimaerism rate of *B.01* in R188 was determined to be 5.30% (174 copies/20 μl well), confirming the presence of chimaerism (Figure 4d). In the case S739, the ddPCR revealed an imbalance of haplotypes (29.5% *B* vs. 70.5% *O*, Figure 4c), which significantly deviates from normal *B/O* control (52.3% *B* vs. 47.7% *O*, Figure 4f), and distinguished the individual with *B/O-O/O* chimaera from people of certain B subgroups with mixed field agglutination reaction (*B/O* genotype without any mutation).

DISCUSSION

Pruss et al. found that a chimaerism ratio as low as 5% could lead to serious complications in transfusion, although the haemolysis could subside within a few hours without special treatment [3, 9]. Being vigilant to the issue of chimaerism and its complications, we generally refused the donor when a blood group chimaera was detected; and when the recipient patient was positive for blood type chimaera, a careful serological cross-matching was conducted to search for compatible blood unit for transfusion.

In this study, molecular techniques such as PCR-SSP/ABO genotyping, ABO gene sequencing, STR analysis, and ddPCR were performed to identify natural ABO chimaera. The ddPCR is the most sensitive approach for the determination of the status of chimaerism; however, it is expensive, time-consuming and technically demanding. So instead of confirming the results obtained by routine tests, the ddPCR was mostly reserved in the current study for the detection of very low-level chimaerism or complicated cases, which could not be identified by other methods. Only 27% of suspected samples were identified as chimaera by PCR-SSP, and the detective rate greatly improved (67%) when ABO gene sequencing was performed. Furthermore, more chimaeras were confirmed by the STR-based method (87%), but STR is not suitable for the assessment of chimaerism in the micro range (<1%) [10]. Therefore, droplet digital PCR was attempted to detect samples for which the previous three molecular biology methods were inconclusive (S114 and S158) or with very weak evidence (S739 and R188); not only for microchimaerism but also for those high-degree chimaeras that were hard to distinguish from some ABO subgroup.

The ddPCR is a very sensitive molecular biological method and is able to detect a copy of mutant DNA in a 100,000-fold excess of wild-type background [11]. In the last decade, ddPCR has been used in tumour genotyping with cell-free plasma DNA with high reliability [12] and was also adopted to evaluate haematopoietic stem cell

transplantation (HSCT) microchimaeric rate after transplantation [10, 13] or maternal microchimaerism [14, 15]. The technique also enabled the identification of maternal microchimaerism with a sensitivity of approximately 1/10,000 [16].

Droplet partitioning reduces competitive amplification effects, allowing detection down to 0.001% mutant fraction, which is 1000 times lower than that with real-time PCR, and thus able to detect extremely low levels of chimaerism. In the current study, with the high sensitivity and specificity of ddPCR, we are able to identify individuals with ABO microchimaerism, with the degree of chimaerism as low as 0.12%.

The detection rate of natural blood group chimaeras could be greatly increased by gradually adopting PCR-SSP/DNA sequencing, STR, and ddPCR; this is also true for microchimaeras, which have not been identified by many current laboratory methods.

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Y.W. and Q.M. performed ABO phenotyping and molecular biology analysis of samples, drafting of this paper. Q.M. and H.H. contributed to the acquisition of data, analysis and interpretation of supplementary ddPCR experiments and critical revision of this work. H.L. and W.Z. performed selection and collection of patients for ABO blood group chimaera identification. X.W. assisted in the completion of the results and critical revision of this work. C.Q. and X.C. provided substantial contributions to study conception and design, and critical revision for this manuscript. This work was supported by the WeiGao Science Foundation of the Chinese Society of Blood Transfusion, WGSF-CSBT (CSBT-WG-2019-01), and the National Natural Science Foundation of China (82000183).

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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SUPPORTING INFORMATION

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

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SHORT REPORT

Vox Sanguinis ISBT International Society of Blood Transfusion

Contamination of platelet concentrates with *Staphylococcus aureus* induces significant modulations in platelet functionality

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Canadian Blood Services and Health Canada

Abstract

Background and Objectives: Platelet concentrates (PCs) contaminated with *Staphylococcus aureus* can escape detection during PC screening, causing septic transfusion reactions. This study aimed to determine the impact of *S. aureus* contamination on platelet metabolism and functionality during PC storage.

Materials and Methods: Targeted metabolomics ($N = 3$) was performed on non-spiked PCs and PCs inoculated with 10–20 colony-forming units (CFU)/bag of *S. aureus*. Metabolites were quantified at 0, 48 and 144 h using high-performance mass spectrometry (MS). Additionally, PCs spiked with approximately 20 CFU/bag of *S. aureus* were sampled every 24 h for up to 144 h to evaluate platelet functionality using flow cytometry ($N = 2$).

Results: Eight metabolites had significantly different levels in spiked PCs (log2 fold-change ≤ or ≥±1) versus non-spiked units at 48 and 144 h. Xanthine, uridine, serine, glutamine and threonine were increased, whereas orotic acid, dihydroorotic acid and aspartic acid were decreased. Flow cytometry showed a significant decrease in expression of GPIIb while P-selectin expression was significantly increased in spiked PCs after 72 h of storage when *S. aureus* concentration was ≥10E+08 CFU/ml. Additionally, phosphatidylserine exposure was significantly increased after 48 h of PC storage, when *S. aureus* had reached a concentration of 2E+06.

Conclusion: Contamination with *S. aureus* exacerbates platelet storage lesions in contaminated PCs but only when the bacterium has reached clinically significant levels.

Keywords

bacterial contamination of platelets, platelet flow cytometry, platelet metabolomics

Highlights

- Platelet concentrates contaminated with high levels of *S. aureus* undergo significant metabolomic changes during storage.
- Clinically significant concentrations of *S. aureus* exacerbate platelet activation and exposure of phosphatidylserine in contaminated platelet concentrates.
- Bacterial contamination has an impact not only on safety but also on the quality of platelet concentrates by affecting platelet functionality.

INTRODUCTION

Platelet concentrates (PCs) are used to treat thrombocytopenic patients and prevent bleeding during surgical interventions. Storage of PCs at 20–24°C under agitation is important to maintain platelet functionality [1]. Unfortunately, these conditions allow contaminant bacteria, introduced during venipuncture, to potentially proliferate and reach clinically significant levels (≥1.00E+0.5 colony-forming units (CFU)/ml) [2], posing a safety threat to transfusion patients [2]. More recently, Gammon et al. have shown that bacteria can also be introduced after PC production if the integrity of the storage container is compromised [3]. Organisms such as *Staphylococcus aureus*, with slow growth rates and/or able to form surface-attached aggregates known as biofilms in PCs, can escape detection during PC screening with culture systems [4, 5]. *S. aureus* is a predominant pathogen causing septic transfusion reactions involving contaminated PCs worldwide [4, 5].

Metabolomics has emerged as a powerful tool to understand mechanisms underlying physiological processes and diseases [6]. Paglia et al. used metabolomics to decipher platelet metabolome modulations during PC storage, showing that metabolic decay does not occur linearly, and two discrete phenotypes are displayed by apheresis and buffy coat PCs [7]. Recently, a metabolomics approach was employed to compare the metabolic phenotypes of PCs stored under standard conditions and cold-stored PCs [8]. To our knowledge, this is the first study aimed at determining the impact of bacterial contamination on platelet metabolism and functionality during PC storage.

MATERIALS AND METHODS

Ethical approval for this study was granted by the Canadian Blood Services Research Ethical Board. Apheresis PC units were collected at the Canadian Blood Services netCAD Blood4Research Facility (netCAD, Vancouver, Canada) in agreement with standard procedures. PCs and plasma were shipped to the Canadian Blood Services Microbiology laboratory in Ottawa, Canada. PCs were screened for bacterial contamination upon arrival using established protocols [9]. PC units were split into two, each with approximately 200 ml. One of the split units was used as a control (non-spiked), while the other one was inoculated with 10–20 CFU/bag (metabolomics) or 20 CFU/bag (flow cytometry) of *S. aureus* CBS2016-05, a strain involved in a septic transfusion reaction [4].

For metabolomics studies, 50 µl of PC samples from control and spiked units were collected at 0, 48 and 144 h of storage in pre-chilled 2 ml tubes, followed by the addition of 600 µl of ice-cold metabolite extraction buffer MeOH:H2O:ACN: (1:1:1, v/v/v) and kept in −80°C since it has been shown that metabolites stored at this temperature are stable for up to 30 months [10]. Samples were then thawed, and cell lysis was done by double bead beating for 60 s at 3584*g* followed by incubation with a 2:1 dichloromethane:water solution on ice for 10 min. The polar and non-polar phases were separated by centrifugation at 4000*g* for 10 min at 1°C. The upper polar phase was dried using a refrigerated CentriVap Vacuum Concentrator at −4°C (LabConco Corporation, Kansas City, MO). Samples were resuspended in water and run on an Agilent 6470A tandem quadruple mass spectrometer (MS) equipped with a 1290 Infinity II ultra-high-performance liquid chromatograph (Agilent Technologies) utilizing the Metabolomics Dynamic MRM Database and Method (Agilent), which uses an ion-pairing reverse phase chromatography. This method was further optimized for phosphate-containing metabolites with the addition of 5 µM InfinityLab deactivator (Agilent) to mobile phases A and B, which requires decreasing the backflush acetonitrile to 90%. Multiple reaction monitoring (MRM) transitions were optimized using authentic standards and quality control samples. Metabolites were quantified by integrating the area under the curve of each compound using external standard calibration curves with Mass Hunter Quant (Agilent). Assays were repeated with three different PC units.

For flow cytometry analyses, PC samples from spiked and non-spiked units were analysed for platelet counts using a Sysmex pocH-100i™ Automated haematology analyser mode (Sysmex Corporation, Kobe, Hyogo, Japan). PC samples were treated with phycoerythrin (PE)-labelled CD62P (P-selectin) antibody as a platelet activation marker, PE-labelled CD41a (GPIIb) antibody as a platelet marker, and Annexin V binding to evaluate exposed phosphatidylserine moieties on the platelet membrane. Samples were diluted with phosphate-buffered saline (PBS) or Annexin binding buffer to a concentration of 10–40 $\times$ 10^6 platelets/ml. A 50 µl volume of diluted platelets was incubated with 10, 5 and 1.5 µl of the assay reagents in a final volume of 100 µl adjusted with the respective buffer, and then stored at room temperature for 20 min in the dark. A 400 µl volume of PBS or Annexin binding buffer was added to each sample before analysis on an Attune acoustic focusing cytometer equipped with 488 and 637 nm lasers (Life Technologies, ThermoFisher Scientific, Waltham, MA, USA). Flow cytometry assays were done in duplicate.

Statistical analyses were performed in MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/). Metabolite abundances were normalized using log-transformation and subjected to Pareto scaling. Fold changes were calculated for metabolites in spiked samples versus control samples. Hierarchical clustering was performed using Euclidian distance and Ward's method. *t*-test was used to compare the expression of P-selectin and GPIIb, and exposure of phosphatidylserine in control versus spiked PCs. $p \leq 0.05$ was considered significant.

RESULTS

Metabolite expression was not different between control and spiked PCs at 0 h; however, significant modulations were observed in metabolite concentrations in spiked PCs compared to non-spiked PCs at 48 and 144 h of storage when bacteria had reached concentrations of 1.69E+07 and 2E+10 CFU/ml, respectively. These massive changes in metabolites were observed in major metabolic pathways: TCA cycle, glycolysis, amino acids, pentose phosphate pathway, organic acids and nucleotides. At these time points, 76 metabolites were quantified, among which 15 and 48 showed differential changes in concentrations at 48 and 144 h, respectively (log2 fold-change ≤ or ≥±1, $p < 0.05$) (Figure 1a,b). Combined data from spiked PCs revealed

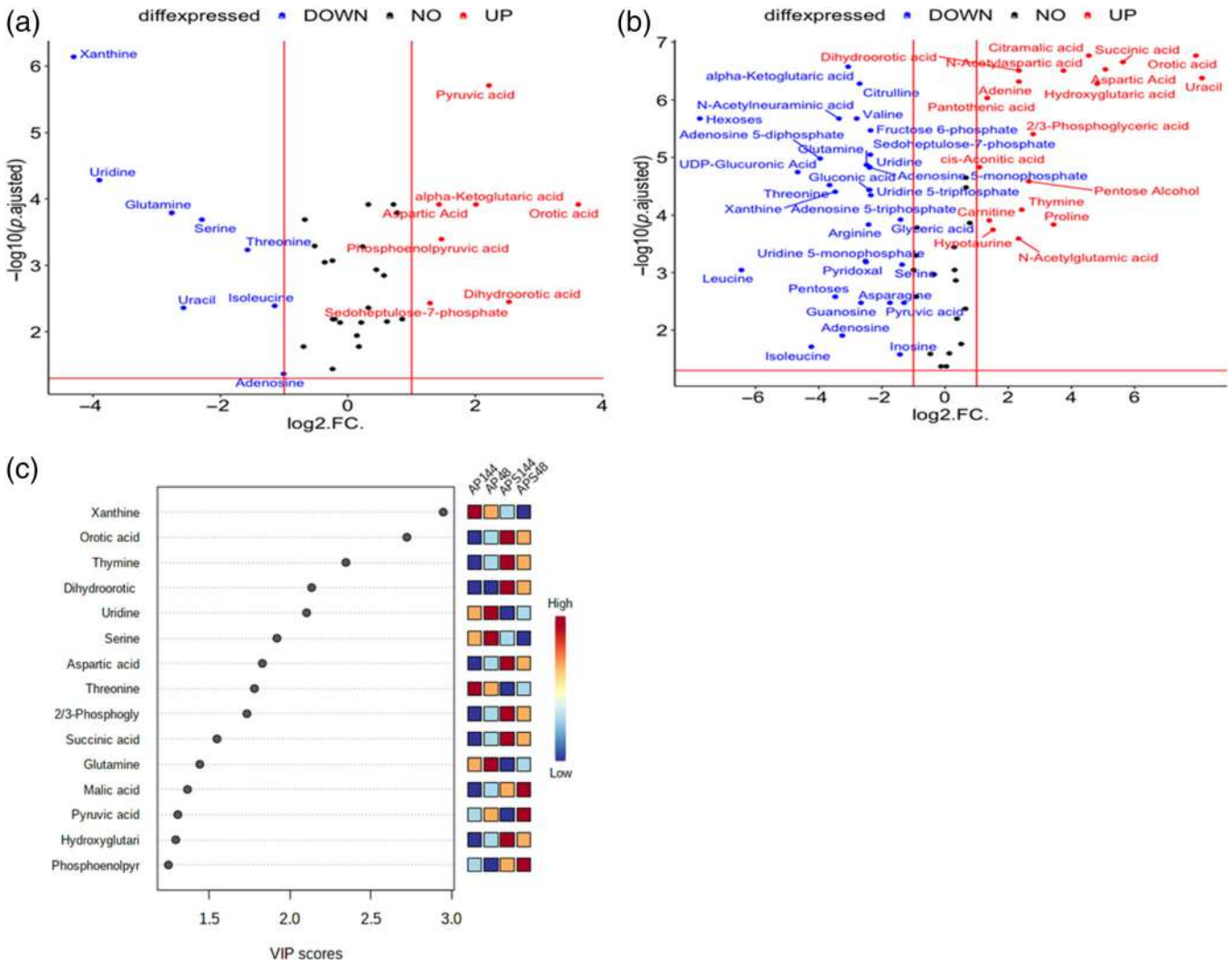

FIGURE 1 Volcano plots were generated based on log2 fold change (FC) between platelet concentrates (PCs) spiked with *Staphylococcus aureus* CBS2016-05 versus non-spiked PC samples at (a) 48 h post-spiking and (b) 144 h post-spiking. Plots highlight differential metabolite concentrations (log2 fold-change ≤ or ≥±1, $p < 0.05$) indicated by red lines. (c) PLS-DA's VIP scores show metabolites that differ significantly between PCs spiked with *S. aureus* CBS2016-05 and non-spiked PCs. AP48 and AP144 refer to control (non-spiked PC samples taken at 48 and 144 h, respectively), while APS48 and APS144 refer to samples of spiked PC taken at 48 and 144 h, respectively. $N = 3$

15 common metabolites with a differential concentration between spiked and control PCs. Within these 15 metabolites, the concentration of eight metabolites differed significantly (log2 fold-change ≤ or ≥±1, $p < 0.05$) between control and spiked PCs at both sampling time points. These included decreased levels of xanthine, uridine, serine, glutamine, and threonine and enhanced concentrations of orotic acid, dihydroorotic acid and aspartic acid in spiked PCs compared to control (Figure 1c). Principal component analysis revealed that the non-spiked control PCs clustered together while spiked PCs samples showed a clear separation (Figure S1).

Flow cytometry analyses summarized in Table 1 showed three important changes in platelet activation and function in *S. aureus* spiked PCs in comparison to control units: (1) a similar percentage of GPIIb expression in control and spiked PCs was observed up 72 h of storage; however, after 72 h, when bacterial concentration was 6E+08 CFU/ml, GPIIb significantly decreased from approximately 99% to about 79% in spiked PCs ($p = 0.02$), and it further decreased to approximately 61% after 144 h of storage; (2) a significant enhancement in the percentage of P-selectin expression (i.e., CD62P+ platelets) was also observed in spiked PCs compared to control units after 72 h of storage (approximately 17% vs. 63% $p = 0.01$); however, a difference in P-selectin expression was not observed at 144 h of storage; and, (3) a significant increase of phosphatidylserine+ platelets in spiked PCs in comparison to control units started after 48 h of storage ($p = 0.04$) when bacterial concentration was 2E+06 CFU/ml, and continued until the end of storage.

DISCUSSION

This study explored the metabolic profile of bacterially contaminated PCs. The effect of *S. aureus* on platelet metabolism and

TABLE 1 Flow cytometry analysis of non-spiked platelet concentrates (PCs; control) and PCs spiked with *Staphylococcus aureus* CBS2016-05 [3] at different time points during PC storage ($n = 2$)

Sample collection time	GPIIb expression		P-selectin expression		Phosphatidylserine exposure		
	%	*p*-value	%	*p*-value	%	*p*-value	CFU/ml
Control 0 h	99.4 ± 0.2	0.083	7.0 ± 0.4	0.500	1.1 ± 0.1	0.626	NA
Spiked 0 h	98.2 ± 0.1		7.6 ± 0.6		1.3 ± 0.3		<1
Control 24 h	99.2 ± 0.3	1.000	15.9 ± 0.6	0.519	2.2 ± 1.0	0.425	NA
Spiked 24 h	99.2 ± 0.1		14.3 ± 3.1		1.3 ± 0.1		10–20
Control 48 h	99.2 ± 0.3	0.058	12.0 ± 0.3	0.086	1.6 ± 0.1	0.039	NA
Spiked 48 h	98.6 ± 0.2		14.2 ± 0.2		4.0 ± 0.1		2E+06
Control 72 h	99.1 ± 0.1	0.017	17.0 ± 3.9	0.011	2.3 ± 0.3	0.006	NA
Spiked 72 h	78.8 ± 0.9		63.6 ± 1.3		54.7 ± 0.4		6E+08
Control 144 h	99.1 ± 0.1	0.002	24.1 ± 1.9	0.263	3.0 ± 0.7	0.008	NA
Spiked 144 h	61.4 ± 0.2		30.5 ± 2.1		78.8 ± 2.1		9E+09

Abbreviations: CFU, colony-forming units; NA, not applicable.

functionality was dependent on bacterial density, as the bacterial concentrations, which showed an impact on platelet metabolome and function, were 1.69E+07 and 2E+06 CFU/ml, respectively. Interestingly, xanthine was one of the metabolites found at significantly lower concentrations in spiked PC samples (Figure 1). This metabolite has been shown to decrease during PC storage as a result of platelet storage lesions (PSL). Acceleration of PSL in spiked PC units was confirmed with flow cytometry analysis, which showed a significant loss in GPIIb expression and increased CD62P+ and phosphatidylserine+ platelets at high bacterial concentrations. Interestingly, at 144 h of storage, when *S. aureus* concentration had reached 9E+09 CFU/ml (Table 1), no differences in the percentage of P-selectin expression were observed between control and spiked units suggesting that *S. aureus* accelerated PSL, likely resulting in shedding of P-selectin.

Our studies also showed that glutamine was significantly decreased in contaminated PCs in comparison to control units (Figure 1), and Paglia et al. have shown that secretion of this metabolite decreases during PC storage [7]. Glutamine is a preferential substrate for mitochondrial oxidative processes and is thus actively metabolized by human platelets [11]. Significant depletion in glutamine concentration in spiked PCs suggests the dysfunctionality of mitochondrial activity. Similarly, in our studies, succinic acid was one of the metabolites found at higher concentrations in spiked PC samples compared to control units (Figure 1). The secretion of this metabolite has been previously linked to the loss of platelet mitochondrial function [12]. As optimal mitochondrial activity is essential for platelet function [13], our observations merit further investigation of mitochondrial function in the context of PC contamination with bacteria.

In our study, metabolites could be originated from platelets or bacteria; however, there was a marked difference between the metabolome of control PCs versus spiked units. Hence, our results demonstrate that PC contamination with *S. aureus* leads to substantial changes in the metabolite content of PCs and accelerates PSL. Although further studies are needed to investigate the impact of other strains of *S. aureus* and different bacterial species on platelet metabolome and function, we have provided preliminary evidence that bacterial contamination not only has an impact on PC safety but also on platelet quality and functionality. Additionally, metabolomic changes in PCs could be used as an indicator of high levels of contamination, which are often linked to septic transfusion events. It would be interesting to evaluate the clinical impact of our findings. One option could be measuring platelet microparticles in contaminated PC units, which have been associated with reduced platelet recovery post-transfusion [14].

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This study was conceived and led by B.Y. and S.R.A.; N.P. and R.P. provided guidance for the experimental design and interpretation of results of the flow cytometry analyses, while experimental work was performed by B.Y. Data were collected and analysed by B.Y., S.R.A. and R.P. The manuscript was written by B.Y. and S.R.A. and reviewed by N.P. and R.P.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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SUPPORTING INFORMATION

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

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SHORT REPORT

Vox Sanguinis ISBT International Society of Blood Transfusion

Whole blood transfusion and paroxysmal nocturnal haemoglobinuria meet again: Minor incompatibility, major trouble

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Abstract

Background and Objectives: The field of transfusion medicine started out with whole blood transfusion to treat severe anaemia and other deficiencies, and then transitioned to component therapy, largely leaving the practice, and experiences, of whole blood transfusions behind. Currently, the field is circling back and whole blood is gaining ground as an alternative to massive transfusion protocols.

Materials and Methods: Herein we describe a severely anaemic paroxysmal nocturnal haemoglobinuria (PNH) patient initially suspected of suffering from renal haemorrhage, receiving a standard low-titre group O whole blood transfusion during pre-hospital transportation.

Results: Following the transfusion, the patient suffered a clinically unmistakable haemolytic transfusion reaction requiring supportive treatment in the intensive care unit. Clinical observations are consistent with an acute haemolytic reaction. The haemolysis was likely due to minor incompatibility between the plasma from the transfused whole blood and the patient's PNH red cells. Recovery was uneventful.

Conclusion: This revealed an unappreciated contraindication to minor incompatible whole blood transfusion, and prompted a discussion on the distinction between whole blood and erythrocyte concentrates, the different indications for use and the importance of emphasizing these differences. It also calls attention to patient groups where minor incompatibility can be of major importance.

Keywords

blood components, haemolytic transfusion reaction, haemovigilance, transfusion reactions, transfusion therapy

Highlights

- Low-titre group O whole blood transfusion is not safe for all patients.
- Minor ABO incompatibility can be of major importance in patients with paroxysmal nocturnal haemoglobinuria.

- These issues should be addressed when transitioning from erythrocyte concentrates to whole blood in pre-hospital emergency services.

INTRODUCTION

During the 1970s and 1980s, civilian transfusion services transitioned from whole blood transfusion to component therapy; both to offer more tailored treatment for specific deficiencies and to better utilize donated units [1]. Component therapy has since been the rule, while the practice of whole blood transfusion has stood sanguinely by, before being picked up by military services. Leaning heavily on war-zone experiences, whole blood transfusion is again on offer in some locations and its use is increasing.

Whole blood offers a convenient product when it comes to administration and is, at least theoretically, a more physiologically sound product with less dilutive additive solutions than a combination of erythrocytes, plasma and platelets. A recent report showed improved overall survival and decreased blood component use when whole blood is used in trauma patients [2], but meta-analyses [3, 4] have failed to show superiority of whole blood compared to balanced massive transfusion. When excluding the logistical benefits, there is no definite evidence in favour of whole blood transfusion.

Transfusing group O whole blood results in non-group O patients receiving incompatible plasma. To reduce the risk of haemolysis, low-titre units are used. There is no clear definition of low titres, and centres offering low-titre group O whole blood (LTOWB) report a range of accepted titres and techniques to obtain said titres [5]. In our region low titre is defined as anti-A and anti-B IgM and IgG titres <256. A study of patients receiving ≥4 units of whole blood comparing haemolytic markers between non-group O and group O recipients, found no evidence of haemolysis [6], and LTOWB is considered safe for recipients of all ABO blood types.

However, as whole blood returns to transfusion practice, its potential benefits and risks for non-trauma patients who might receive it should be evaluated, as we were reminded following one of the first whole blood transfusions in our region.

CASE

A middle-aged man with a history of aplastic anaemia, PNH and renal carcinoma presented to primary care complaining of extreme fatigue and generalized pain. Details of the patient's haematological medical history were unknown to the treating physician, but there was a note of previous admissions due to septicaemia.

Upon presentation, he was afebrile and clinically stable, with normal blood pressure (143/66 mmHg) and slightly elevated pulse rate of 91 bpm. The physician noted pallor, jaundice and dark urine. Haemoglobin was 4.6 g/dl, and the clinical suspicion was septicaemia and anaemia secondary to ongoing renal haemorrhage.

Due to long transportation distance, the patient was sent by air ambulance to the nearest hospital. As haemorrhage was suspected, he received one unit of LTOWB and tranexamic acid. He was clinically stable during transportation.

On arrival, he was still stable; blood pressure 184/89 mmHg, pulse 79 bpm and a slightly elevated respiratory rate of 20. Haemoglobin was 5.1 g/dl. Blood type was A RhD positive, antibody screen negative and direct antiglobulin test (DAT) positive. His on-record blood type, however, was A RhD negative, and the reaction with anti-D was explained by transfused O RhD positive whole blood. The blood bank noted dark plasma and suspected haemolysis. They contacted their transfusion consultant, who was aware of the patient's underlying haemolytic condition. The patient had suffered PNH-related breakthrough haemolysis on several occasions, presenting with haemolytic anaemia requiring transfusion treatment. All prior antibody screens and DATs were negative and transfusion episodes uneventful. On admission the patient was stable, and taken together with the now recognized prior history and dark urine before the transfusion, the consultant found no immediate reason to suspect a reaction to the transfused blood.

However, unbeknownst to the blood bank, 1 h after admission the patient underwent rapid clinical deterioration with severe confusion, intense back pain, nausea, hypotension (systolic pressure 80 mmHg) and generalized skin erythema. He was transferred to the intensive care unit and treated with fluids, adrenaline, antihistamines, morphine and steroids for a suspected transfusion reaction. Haemoglobin had declined to 4.4 g/dl.

Haemolysis was confirmed by haemoglobinuria, dark plasma, elevated lactate dehydrogenase (LDH) and bilirubin, and low haptoglobin (Table 1). There were no values available from before the transfusion, and the contribution of each haemolytic process (underlying vs. acute) could not be established. As the blood bank was unaware of the reaction until the following day, the implicated product was no longer available, and a full transfusion reaction work-up, including cross-matching and cultures could not be completed. Both the patient and the donor were antibody screen negative, blood cultures negative and clinical observations are consistent with an acute haemolytic reaction.

After the acute treatment, the patient stabilized and eculizumab was infused followed by four units of A RhD negative erythrocyte concentrates. Haemoglobin level rose to 8.4 g/dl, bilirubin and creatinine normalized, while LDH decreased. Haptoglobin levels remained low throughout the 5-day admission period.

Three months later, he again suffered from anaemia after PNH-related breakthrough haemolysis requiring transfusion treatment, this time uneventfully with ABO-identical erythrocyte concentrates. The antibody screen was still negative.

TABLE 1 Relevant laboratory measurements during admission

	↓	Admission	Day 1	↓	Day 2	↓	Day 3	Day 5
Haemoglobin, g/dl (13–17)		5.1	4.1		5.7		8.5	8.4
Lactate dehydrogenase, U/L (105–205)		[a]	[a]		3174		2241	1285
Bilirubin, μmol/L (5–25)		57	47		26		24	-
Creatinine, μmol/L (60–105)		155	159		125		111	94
Haptoglobin, g/L (0.3–2)		<0.3	-		<0.3		-	-

Note: The admission values are *after* whole blood transfusion and *before* the clinical reaction. The initial clinical presentation is consistent with already ongoing haemolysis causing anaemia, and though the patient suffered a clinically obvious acute haemolytic transfusion reaction, the contribution of the whole blood transfusion on the laboratory parameters cannot be established. ↓ Denotes transfusion.
[a]Denotes haemolysis interference.

DISCUSSION

PNH is an acquired haematopoietic disorder characterized by intravascular haemolysis, thrombosis and fatigue. It originates from clonal haematopoiesis, where a phosphatidylinositol glycan class A mutated clone gives rise to daughter cells having reduced expression of or lacking glycosylphosphatidylinositol-anchored proteins. For red cells, this translates to a deficiency of complement regulatory proteins, most notably CD55 (decay-accelerating factor) and CD59 (membrane inhibitor of reactive lysis) rendering them vulnerable to complement-dependent haemolysis [7]. Before the introduction of complement inhibitors, treatment options were limited and haemolysis was an overwhelming problem. The resulting anaemia was treated by transfusion, and as haemolytic complications were frequently encountered [8], washed cellular products were recommended for many years [9, 10]. A retrospective study done on PNH patients transfused with various blood products over a 38-year period concluded that washing is not necessary and that the use of type-specific cellular products was safe [9], and currently no specific transfusion recommendations exist. Current literature addressing transfusion in PNH focuses on frequencies of red cell transfusion in the eculizumab era [11, 12].

Eculizumab, a C5-inhibitor, has revolutionized PNH treatment. By C5 complement inhibition, membrane attack complex-assembly followed by intravascular haemolysis is markedly decreased, resulting in reduced transfusion needs and improved quality of life for PNH patients [13]. Breakthrough haemolysis does occur, both intravascular at the end of the dosing interval, and extravascular due to C3-deposition on the erythrocyte surface, yet overall eculizumab is an effective treatment [14]. The reduced need for transfusions, combined with the use of erythrocyte concentrates with minimal amounts of plasma and the protection offered by eculizumab, makes haemolytic transfusion reactions a rare event in this group.

For this patient, the latest flow cytometric quantification of PNH populations 10 months earlier showed 99% Type III cells among granulocytes and monocytes. As he was recently transfused at the time, erythrocytes were 65% Type III cells and 34% Type I cells. PNH-population quantification was done by assessing the level of fluorescent aerolysin (FLAER) and CD24-expression on CD15-gated granulocytes, level of FLAER and CD14-expression on CD64-gated monocytes and level of CD59 expression on CD235a-gated erythrocytes. He received treatment with biweekly eculizumab infusions. Unfortunately, before the described event, it was 3 weeks since the last dose. As whole blood was infused, donor-derived anti-A IgM, though in low titres, bound to the patient's erythrocytes initiating antibody-mediated complement activation. Without the protection of complement inhibitors, natural or supplied, the patient's erythrocytes haemolysed, and the clinical description encapsulates a classic acute haemolytic transfusion reaction.

One might speculate that complement from the transfused whole blood contributed to the deterioration, as endogenous complement factors might have been at least partially consumed in the already ongoing haemolysis. Increasing levels of complement split products have been found in whole blood during storage [15], and on this note, there are other patient groups that probably should not receive plasma-rich products. Patients with cold agglutinin disease have a steady state of depleted C3 and C4, and infusion of plasma could worsen haemolysis [16].

PNH cells are exquisitely sensitive to haemolysis, a property utilized in assessing haemolytic potential of alloantibodies and the effects of complement inhibition [17]. In an in vitro model using 2-aminoethylisothiouronium treated cells to mimic PNH, investigators [18] showed the potential problems of minor incompatibilities in patients with complement inhibitory protein defects, thus making an in vitro prediction of the outcome of a minor incompatible whole blood transfusion to a PNH patient. As the haemolytic potential depends on clone size, patients with smaller PNH clones will probably not experience such a pronounced reaction, yet still present with biochemically evident haemolysis after transfusion.

As whole blood transfusion subsided and PNH patients receive treatment offering protection from complement-mediated haemolysis, thus reducing transfusion needs, knowledge of well-known complications when transfusing PNH patients with out-of-group plasma-rich products is evanescing. We were reminded as one of the first pre-hospital whole blood transfusions was given to a sub-optimally treated PNH patient. Non-group O PNH patients now have a note in the regional transfusion information technology (IT) system to avoid transfusions resulting in minor incompatibility.

This illustrates the need to emphasize that whole blood and erythrocyte concentrates are not interchangeable products for all patients when transitioning from one to the other in pre-hospital

services. One must call attention to the difference plasma makes and show restraint in transfusing anaemic non-bleeding patients with whole blood.

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CONFLICT OF INTEREST

The authors disclose no conflicts of interest.

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SHORT REPORT

Vox Sanguinis ISBT International Society of Blood Transfusion

Fatal haemolytic transfusion reaction due to anti-En^a and identification of a novel *GYPA* c.295delG variant in a Thai family

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Abstract

Background and Objectives: High-frequency antigen En^a (MNS 28) is expressed on glycophorin A (GPA). En(a−) individuals can form anti-En^a when exposed to GPA. A Thai patient formed an antibody that reacted against all reagent red blood cells (RBCs). The patient received incompatible blood resulting in a fatal haemolytic transfusion reaction (HTR). This study aimed to characterize the antibody detected in the patient and investigate the cause of HTR.

Materials and Methods: Blood samples from the patient and three of his family members were investigated. Massively parallel sequencing (MPS) and DNA-microarray were used for genotyping. Standard haemagglutination techniques were used for phenotyping and antibody investigations.

Results: DNA sequencing showed the patient was homozygous for *GYPA*M* c.295delG (p.Val99Ter) predicting En(a−). Three family members were heterozygous for *GYPA* c.295delG. MPS and DNA-microarray predicted the patient was N− discordant with the N+ RBC phenotype. The patient's plasma was positive with enzyme/chemical-treated reagent RBCs but failed to react with En(a−) and M^{k}M^k RBCs.

Conclusion: The *GYPA* c.295delG variant prevented GPA expression on RBCs resulting in En(a−) phenotype. The N+ phenotype result was probably due to the anti-N typing reagent detecting 'N' (MNS30) on GPB. The patient's alloantibody has anti-En^a specificity.

Keywords

anti-En^a antibody, En(a−), En^a (MNS28), haemolytic transfusion reaction, MNS blood group system

Highlights

- Anti-En^a in an En(a−) Thai patient caused a fatal haemolytic transfusion reaction.
- Homozygosity for the novel *GYPA* c.295delG variant gave rise to En(a−) phenotype.

INTRODUCTION

The *GYPA* gene, together with *GYPB* and *GYPE*, resides on chromosome 4q31.21 and controls the MNS blood group system [1, 2]. Cloning and nucleotide sequencing for blood group genes began in the 1980s [3]. The first blood group gene to be sequenced was *GYPA* [3].

GYPA encodes glycophorin A (GPA), the glycoprotein that carries M and N antigens, as well as several high-frequency antigens, including En^a (MNS28) [2, 4]. Structural or singular nucleotide variations in the *GYPA* gene can prevent normal GPA expression leading to En(a−) phenotype, for example En(Fin)—deletion of *GYPA* exons 2–7; *En(UK)*—a *GYP(A−B)* hybrid; *En(IND)*—*GYPA* c.314_315insG (p.Thr106Asnfs*19) [2, 5]. GPA is also absent in M^kM^k phenotype arising from the deletion of *GYPA* exon 2–7, *GYPB* and *GYPE* exon 1 [2]. The En(a−) phenotype has been reported in English, Finnish, French-Canadian, Japanese, Pakistani, and Indian individuals [2, 5, 6]. En(a−) red blood cells (RBCs) are also Wr(b−) [1, 2].

Individuals lacking GPA when exposed to En(a+) RBCs can develop anti-En^a. Anti-En^a is a general term for antibodies with specificity against the external region of GPA [1, 2]. Based on the effect of proteolytic enzymes on GPA, the serological reactivity of anti-En^a can be classified as anti-En^aTS (trypsin-sensitive), −En^aFS (ficin-sensitive), and −En^aFR (ficin-resistant) [1, 2]. Anti-En^a can be naturally occurring [6]. Auto-anti-En^a has been associated with autoimmune haemolytic anaemia (AIHA) [1, 2]. Patients treated with pembrolizumab have been reported to form red cell antibodies with anti-En^a specificity causing AIHA [7, 8]. Allo-anti-En^a has been reported to cause haemolytic disease of the foetus and newborn (HDFN) and haemolytic transfusion reaction (HTR) [2].

CASE PRESENTATION

A Thai male patient was diagnosed with myelodysplastic syndrome and acute myeloid leukaemia and received treatment at a hospital from October 2020 to December 2020. It was not known if the patient was transfused at this hospital.

Four months later, in April 2021, the patient was admitted (first admission) at our hospital and was indicated for blood transfusion. Pre-transfusion testing showed the patient was Group A, D+ and antibody screen positive. Anti-Di^a and anti-S were identified, and extended phenotyping was performed (Table 1). The patient was phenotype-matched for S−, Mi(a−), Di(a−), Fy(b−) and K− packed red blood cells (PRBC) units. From April 2021 to August 2021, the patient received 51 compatible units using a polyspecific antihuman globulin column agglutination test (CAT). Anti-HI and auto-anti-Jk^b were detected after transfusion with a few PRBC units.

A timeline of the patient's haemoglobin levels (June 2021–September 2021) is shown in Figure 1. The patient's haemoglobin level dropped to 5.6 g/dl (25 August 2021), and a pan-agglutinating antibody was detected (3+ reaction) in indirect antiglobulin test (IAT) using CAT while performing pre-transfusion crossmatch on the 52nd PRBC unit.

TABLE 1 Patient's blood type

Observed phenotype (serology): Pre-transfusion sample	C+E+c+e+ **M−N+** S−s+ Mi(a−) P1+ Le(a−b+) Jk(a+b+) Fy(a+b−) K−k+ Di(a−)
Predicted phenotype (ID CORE XT genotyping)	C+E+c+e+ Cw− V− VS− hrS+ hrB+ K−k+ Kp(a−b+) Js(a−b+) Jk(a+b+) Fy(a+b−) **M+N−** S−s+ U+ Mi(a−) Di(a−b+) Do(a−b+) Hy+ Jo(a+) Co(a+b−) Yt(a+b−) Lu(a−b+)

The patient was randomly crossmatched with phenotype-matched donors, but all units were incompatible. He was transfused with two incompatible units (grading 3+ IAT with a negative auto control). Consequently, he developed a fever and chills during the transfusion.

On 1 September, his haemoglobin level dropped to 4.4 g/dl, and another incompatible unit was given, resulting in a slight haemoglobin increase to 4.7 g/dl 22 h post-transfusion but then decreased to 3.7 g/dl 29 h later. A further two incompatible units were transfused, but no increase in haemoglobin level was achieved. A non-specific coagulation defect was observed in the patient.

He was transfused again with three incompatible units. His haemoglobin level was 4.9, 3.3 and 3.4 g/dl after each respective transfusion. The patient's plasma sample, collected on 6 September 2021, was sent to the Australian Red Cross (ARC) Lifeblood for antibody identification. The patient received the last unit of 8 PRBCs on 8 September 2021 and passed away 48 h later. Laboratory investigations performed before the patient's death, including urine examination, showed haematuria and 2+ bilirubin. Blood tests showed elevated blood urea nitrogen (99 mg/dl; NR: 7–20 mg/dl), creatinine (4.88 mg/dl; NR: 0.5–1.0 mg/dl), and total bilirubin (39.8 mg/dl; NR: 0.2–1.2 mg/dl). All test results supported haemolytic symptoms. This study aimed to characterize the antibody detected and investigate the cause of HTR.

MATERIALS AND METHODS

Study samples

Four samples (father, mother, sister, and patient) were investigated in this study. Phenotyping, enzyme and chemical studies and blood group genotyping by microarray (ID CORE XT, Progenika/Grifols) were performed at the National Blood Centre (NBC), Thai Red Cross Society. Genotyping by DNA sequencing was performed at the Red Cell Reference Laboratory, ARC Lifeblood. This study has ethics approval from the NBC, Thai Red Cross Society Ethics Committee (NBC 2/2022).

Haemagglutination tests

Standard haemagglutination tests were performed by using the conventional tube test and column agglutination technology (LISS/Coomb's Polyspecific IgG/C3d, Ortho clinical diagnostic, NJ, USA)

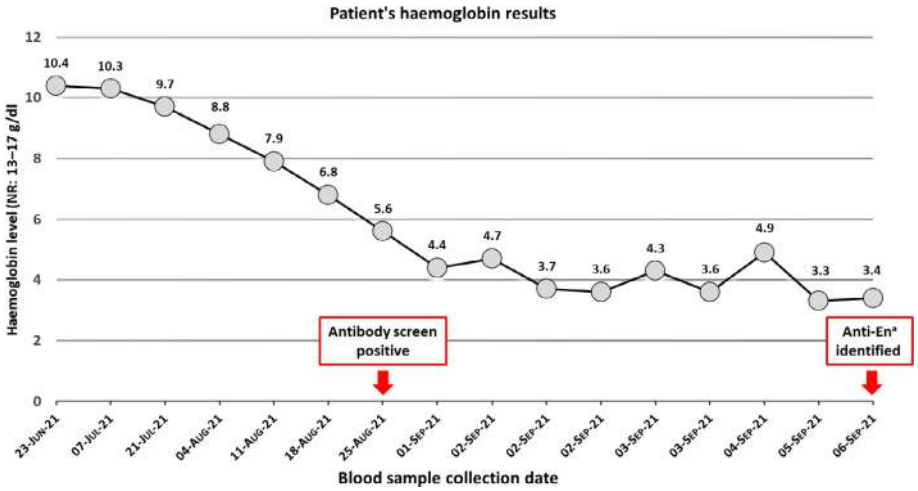

FIGURE 1 Timeline of patient's haemoglobin levels. This graph shows the patient's haemoglobin level steadily decreasing before and during the development of a pan-agglutinating antibody where the patient received incompatible RBC transfusions.

following the manufacturer's recommendation. Phenotyping reagents anti-C, −E, −c, −e, −K, −Jk^a, −Jk^b, −Fy^a, −Fy^b, −Le^a, −Le^b (Diamed GmbH, Cressier FR, Switzerland), anti-k (Immulab, ParagonCare, Victoria, Australia) anti-S, −s (CE-Immunodiagnostika GMBH, Eschelbornn, Germany) were used according to manufacturer's instructions. In-house anti-M, −N, −Mi^a, −P1 monoclonal antibody reagents (National Blood Centre, Thai Red Cross, Bangkok, Thailand) were also used for phenotyping. In-house panel cells (National Blood Centre, Thai Red Cross, Bangkok, Thailand) treated with papain, trypsin, α-chymotrypsin, dithiothreitol (DTT) and aminoethylisothiouronium (AET) (Sigma-Aldrich, Darmstadt, Germany) were tested with patient's plasma to investigate antibody specificity.

Genotyping by microarray

The patient's genomic DNA was isolated from EDTA-whole blood samples using a DNA extraction kit (EZ1 DSP DNA Blood Kit, QIAGEN, Hilden, Germany) in an automated robotic instrument (QIAGEN) as per the manufacturer's instructions. Genotyping using ID CORE XT kit (Progenika Biopharma-Grifols, Bizkaia, Spain) was performed as recommended by the manufacturer.

Genotyping by DNA sequencing

DNA extraction equipment (EZ1 Advanced, QIAGEN) was used to extract DNA from EDTA-whole blood samples as recommended by the manufacturer. Massively parallel sequencing (MPS) was performed as previously described [9]. DNA was prepared using the Illumina DNA prep with an enrichment kit (Illumina, San Diego, USA). DNA sequencing was performed on a DNA sequencer (Illumina MiSeq) as per the manufacturer's instructions. Binary alignment map (BAM) files were generated after the reads were aligned to the human reference genome GRCh37/Hg19 on the MiSeq. BAM files were then imported into bioinformatics analysis software (CLC genomics workbench software 20, QIAGEN) to generate annotated variant call files as previously described [9].

RESULTS

RBC phenotyping

The patient's MNS phenotype was M−N+ S−s+ Mi(a−). Extended RBC phenotyping result is shown in Table 1.

Antibody investigation

The patient's plasma was strongly reactive (3+) with all panel cells at IAT using the conventional tube test and column agglutination test and non-reactive with auto control cells. It was incompatible with all RBCs from random blood donors and family members but compatible with En(a−) and M^{k}M^k cells. This reactivity profile suggests that the antibody has anti-En^a specificity. The patient's plasma reacted with all panel cells treated with papain, trypsin, α-chymotrypsin, DTT and AET. This indicates that the epitope, recognized by the antibody, was not destroyed by enzymes and chemicals used in this study.

Allogeneic adsorption of his plasma showed no other alloantibody was present. Previously identified allo-antibodies (anti-Di^a, −S, −HI, and Jk^b) were no longer detected at this stage.

Microarray genotyping

The MNS genotype for the patient predicted M+N−, S−s+U+, Mi(a−). Comparison between phenotype and genotype results

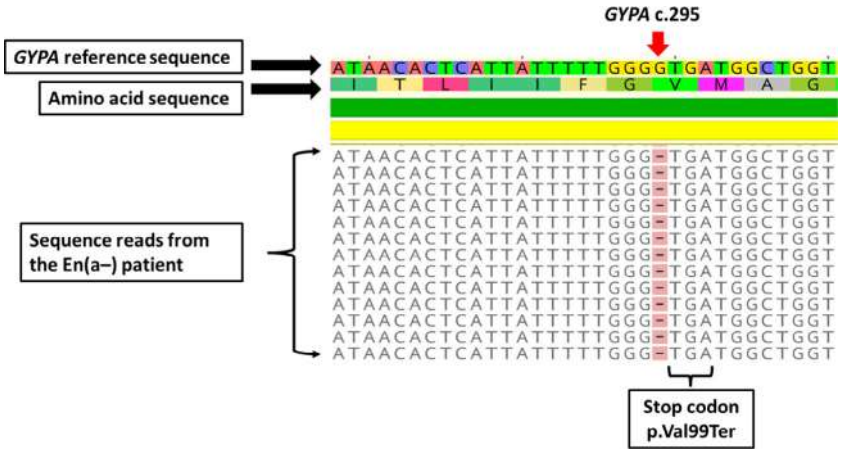

FIGURE 2 *GYPA* exon 5 DNA sequencing alignment. The red arrow shows the location of *GYPA* c.295. Sequence reads from the patient showed a single base G deletion at c.295, represented by a hyphen and highlighted in red. The *GYPA* c.295delG caused a frameshift forming a stop codon (TGA) at amino acid position p.Val99Ter. Sequencing alignment was displayed using Geneious Prime 2022.2.1 (https://www.geneious.com).

showed inconsistent in M/N but consistent in S/s typing (Table 1). Phenotype predictions for other blood groups are shown in Table 1.

DNA sequencing

DNA sequencing by MPS showed that the patient was homozygous for *GYPA*M* predicting M+N−, which is consistent with the microarray phenotype predictions. In addition, MPS detected a novel variant c.295delG, located in *GYPA* exon 5, Figure 2. This variant is expected to form a premature stop codon at p.Val99Ter. The patient was homozygous for c.295delG and is predicted to lack GPA expression. The nucleotide sequence for this novel variant was given accession number GenBank OL860988 (*GYPA*01N.02*). The patient's father, mother and sister were all heterozygous for *GYPA* c.295delG. Consanguinity between the father and mother was not established.

DNA sequencing of the patient's *GYPB* gene predicted S−s+U+, which is consistent with phenotype predictions obtained by microarray. MPS also showed that the patient was homozygous for c.1972G in *SLC4A1* gene (Diego blood group gene) predicting Wr(a−b+) phenotype.

DISCUSSION

This study reports an anti-En^a antibody causing a fatal HTR in a Thai patient. The patient was homozygous for *GYPA*M* c.295delG (p.Val99Ter) that prevented GPA expression and is therefore M−N− En(a−). Individuals lacking GPA can form anti-En^a when exposed to GPA+ RBCs.

Mismatch M and N antigen typing for the patient were observed in this study. First, discordant M antigen typing was identified between two genotyping platforms where MPS predicted M−N−, En(a−), while microarray predicted M+N−. Microarray gave a M+ prediction because it was not designed to detect *GYPA* c.295delG that disrupted the full synthesis of GPA. Second, discordance was observed in the N antigen typing where MPS and DNA-microarray predicted N− while phenotyping gave N+ result. The positive haemagglutination reaction for N was most likely due to the 'N' (MNS 30) present on GPB. The absence of sialic acid-rich GPA made the 'N' (MNS30) epitope on GPB more accessible to this otherwise specific anti-N reagent. This can be proven by treating patient's RBC with α-chymotrypsin to destroy 'N' (MNS30) on GPB. Enyzme-treated RBCs can then be phenotyped using anti-N monoclonal antibody, which is predicted to be negative after treatment with α-chymotrypsin. However, this was not performed because the patient's pre-transfusion RBC samples were no longer available for additional testing. Further sampling was not possible as the patient passed away. The patient was predicted to be Wr(a−b+). Wr^b requires GPA for expression [1]. Since the patient is En(a−), we expected the patient to be Wr(b−). However, we were unable to confirm this phenotype for reasons stated above.

The MNS blood group profile in Thais has unique features. MNS antigens Mi^a, Mt^a and Hop have a prevalence of 9.7%, 0.94% and 0.68%, respectively, in the Thai population, which is higher than other population groups [1, 4, 10]. A rare JENU− phenotype was reported in a Thai individual who develop anti-JENU [11]. Recently, a GP.Mur/GP.Mur Thai mother formed anti-s^D against her baby's s^D+ RBCs causing HDFN [12]. Her serum reacted positive with 2.2% (4/184) Thai blood donors. All four donors carried *GYPB*sD* allele.

In this study, the identification of En(a−) phenotype enhances our understanding about the blood group diversity in the Thai population. To our knowledge, this is the first report of a fatal HTR due to anti-En^a in the Asian population. En(a−) phenotype is extremely rare and national or international blood donor registries would be challenged to identify suitable En(a−) blood donors and provide sufficient blood supply for transfusion in a timely manner. The late identification of the antibody specificity and the clinical disease state of our patient

makes accessing rare donors internationally not a viable option. Other opportunities to provide En(a−) blood are through autologous blood donation and direct donation from family members identified as En(a−).

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P.S., G.H.L. and Y.W.L. wrote the manuscript with contributions from all co-authors. P.S. and Y.W.L. designed the experiments. P.S., M.E., B.W., S.O. and K.L. serological testing. G.M.M. performed D.N.A. sequencing. P.S., G.H.L., S.B. and Y.W.L. reviewed serological and molecular data. S.O. and K.L. provided clinical information. P.S. and G.H.L. created tables and figures. All authors reviewed and approved the final version of this manuscript.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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REPORT

Vox Sanguinis ISBT International Society of Blood Transfusion

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

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Abstract

Background and Objectives: Under the ISBT, the Working Party (WP) for Red Cell Immunogenetics and Blood Group Terminology is charged with ratifying blood group systems, antigens and alleles. This report presents the outcomes from four WP business meetings, one located in Basel in 2019 and three held as virtual meetings during the COVID-19 pandemic in 2020 and 2021.

Materials and Methods: As in previous meetings, matters pertaining to blood group antigen nomenclature were discussed. New blood group systems and antigens were approved and named according to the serologic, genetic, biochemical and cell biological evidence presented.

Results: Seven new blood group systems, KANNO (defined numerically as ISBT 037), SID (038), CTL2 (039), PEL (040), MAM (041), EMM (042) and ABCC1 (043) were ratified. Two (039 and 043) were de novo discoveries, and the remainder comprised reported antigens where the causal genes were previously unknown. A further 15 blood group antigens were added to the existing blood group systems: MNS (002), RH (004), LU (005), DI (010), SC (013), GE (020), KN (022), JMH (026) and RHAG (030).

Conclusion: The ISBT now recognizes 378 antigens, of which 345 are clustered within 43 blood group systems while 33 still have an unknown genetic basis. The ongoing discovery of new blood group systems and antigens underscores the diverse

The views expressed do not necessarily represent the view of the National Institutes of Health, the Department of Health and Human Services, or the US Federal Government.

For affiliation refer to page 1340

and complex biology of the red cell membrane. The WP continues to update the blood group antigen tables and the allele nomenclature tables. These can be found on the ISBT website (http://www.isbtweb.org/working-parties/red-cell-immunogenetics-and-blood-group-terminology/).

Keywords

blood groups, genetics, genomics, red cell antigens, terminology

Highlights

- The ISBT Working Party on Red Cell Immunogenetics and Blood Group Terminology (WP for RCI and BGT) held four business meetings between June 2019 and June 2021 to consider the evidence for ratifying and naming new blood group systems and antigens.
- Seven new blood group systems, KANNO, SID, CTL2, PEL, MAM, EMM and ABCC1, were ratified, and a further 15 antigens added to existing systems: a total of 43 blood group systems comprising 345 antigens are now registered on the ISBT website.
- Significance: The curation of tables for blood group systems, antigens and associated alleles is of clinical significance for accurate typing, and the ISBT WP RCI and BGT members continue to maintain these tables on the ISBT website (https://www.isbtweb.org/isbt-working-parties/rcibgt.html).

INTRODUCTION

The International Society of Blood Transfusion (ISBT) Working Party (WP) for Red Cell Immunogenetics and Blood Group Terminology is responsible for the ratification and naming of blood group systems and antigens. Since the last report, which included developments up to the Toronto meeting in 2018 [1], the WP has held four business meetings. One took place physically in Basel, Switzerland, in conjunction with the 2019 ISBT Regional Congress, and three were held as virtual meetings during the COVID-19 pandemic in 2020 and 2021.

Requests for new blood group systems and antigens were presented at these meetings and accepted according to previously established criteria [2]. Criteria for defining a blood group system include evidence that it is a discrete genetic entity under the control of a single gene or by contiguous homologous genes, in which different antigens within a system are encoded by alternative forms of the gene. All blood group antigens must be defined serologically by a specific antibody formed by a human lacking the antigen in question and shown to be inherited.

Some red cell antigens do not belong to a blood group system because their molecular bases have not been defined. The ISBT WP classifies these antigens into collections or series. There are two series of antigens designated the 700 and 901 series, which represent, respectively, antigens with a prevalence within the population of study of less than 1% or greater than 90%.

This report summarizes the evidence presented in ratifying seven new blood group systems and 15 new antigens over the four meetings. These included antigen(s) previously in the 700 or 901 series, which were transferred into existing or into new blood group systems [2].

NEW BLOOD GROUP SYSTEMS

Seven new blood group systems (designated ISBT 037–043) were registered over the four meetings (Table 1). Only two, CTL2 and ABCC1, comprised de novo antigens, while the other five resolved the genes responsible for expression of previously reported high-prevalence antigens, four formally classified in the 901 series.

System 037: KANNO

An antibody to a high-prevalence antigen, designated KANNO (now KANNO1), was first identified in 1991 in a Japanese woman with a history of pregnancy, although never formally listed in the 901 series [3]. Definitive evidence presented at the Basel meeting showed that KANNO was encoded by the *PRNP* gene at chromosome 20p13. Genome-wide association studies defined single nucleotide variants (SNVs) in four unrelated individuals, with anti-KANNO, which were absent in 415 healthy Japanese. Whole-exome sequencing using 14 unrelated KANNO antigen-negative (KANNO−) individuals then implicated the causal variant, rs1800014 c.655G>A on *PRNP*, resulting in p.Glu219Lys. Family studies supported the genotype and phenotype associations. Monoclonal antibody-specific immobilization of erythrocyte antigen assays showed the KANNO antigen is on the prion protein, as did transfection and expression studies. The *PRNP*655A* variant in the ExAC database has a frequency of 5.8% in Japan, 4% in South and East Asia, 0.03% in Africa and 0.004% in Europe [4].

There is only one antigen in the KANNO system, which is defined as KANNO1 or, under the numerical designation, ISBT 037001 (Table 1). KANNO is named after the first proposita [3].

TABLE 1 New blood group systems registered by the Working Party at business meetings conducted in Basel in June 2019 and over the first virtual meeting in September 2020 and the second virtual meeting in December 2020

Business meeting	ISBT number	Blood group system	Gene name and alternative name	Reference sequence	Antigens	Reference
Basel	037	KANNO	*PRNP* *CD230*	NG_009087.1	KANNO1	[4]
Basel	038	SID	*B4GALNT2* *GALGT2*	AJ517770 and AJ517771	Sd^a	[5]
Basel and first virtual	039	CTL2	*CTL2* *SLC44A2*	NC_000019.10 (KM024996)	VER and RIF	[12]
First virtual	040	PEL	*ABCC4* *MOAT-B*	NG_050651 (LRG 1183)	PEL	[13]
First virtual	041	MAM	*EMP3* *YMP*	(NC_000019.10)	MAM	[15]
Second virtual	042	EMM	*PIGG* *GPI7*	NG_051621.1	Emm	[16, 17]
Second virtual	043	ABCC1	*ABC29*	NG_028268.2	WLF	[20]

Note: *B4GALNT2* has a short and a long form; hence there are two reference sequences. AJ51770 is 60 amino acids longer than AJ517771 due to the differential use of two alternative exons 1. *GALGT2* has been used as an alternative name for *B4GALNT2*.

System 038: SID

The Sd^a antigen was first described in 1967 and classified in the ISBT 901 series as ISBT 901012. The chemical basis of the antigen was known to be a terminal trisaccharide, (GalNAcβ1-4 (NeuAcα2-3)Galβ-R) and a candidate histo-blood group gene, the *B4GALNT2*, chromosomal location 17q21.32, had been cloned in 2003. Nevertheless, the genetic basis for the Sd(a−) phenotype remained unknown. While approximately 10% of Caucasians have Sd(a−) red blood cells (RBCs), only 2%–4% are truly negative and can make anti-Sd^a. The reason is that the Sd^a antigen is also present in other tissues. Against this known background, the evidence presented in Basel, from Sanger sequencing and allele discrimination assays, defined genetic variants in *B4GALNT2* that associate with the Sd(a−) phenotype. The most common variant observed was a variant (rs7224888:C) in exon 10 with an allele frequency of approximately 0.10. This c.1396T>C variant causes a p.Cys466Arg change, and six of nine Sd(a−) individuals were homozygous for c.1396C. One individual was compound heterozygous for rs7224888/rs72835417, the latter being a splice-site variant in intron 8. Another individual was also a compound heterozygote but for low-frequency variants rs148441237 and rs61743617. Finally, one sample had no detectable deviations from the consensus sequence, a finding that warrants further investigation [5]. In addition, an abstract describing another nine Sd(a−) individuals reported eight to be homozygous for c.1396C (rs7224888:C) and one compound heterozygous for rs7224888/rs72835417 [6].

Expression and glycoproteomic studies with *B4GALNT2* constructs with wild-type sequence or the predominant null candidate variant c.1396C were performed in HEK293 cells [7]. While the consensus 'wild-type' allele induced expression of the antigen, the c.1396C construct did not. This variant also resides in a haplotype block associated with a long non-coding (lnc) RNA, and downregulation of this lnc RNA correlates with poor prognosis in cancer, which may be marked by loss of Sd^a. The Sd^a antigen is also involved in the pathogenesis of different infections, and high expression of Sd^a has been correlated to protection against parasite invasion of RBCs [8–10].

The Sd^a antigen was moved from the 901 series (901012) into a new blood group system, SID, ISBT 038 and is the only known antigen in this system (Table 1). SID is one of the eight carbohydrate histo-blood group systems with wide tissue distribution and which depend on genetic variants altering glycosyltransferase activity and/or specificity. The name SID was originally in honour of the head of the maintenance department of the Lister Institute that housed the Race and Sanger laboratory in London, who was the source of the first so-called super-Sid or Sd(a++) control cells [11].

System 039: CTL2

Studies leading to assigning the CTL2 blood group system were initiated by a sample from a pregnant woman who presented with an antibody reacting against a high-prevalence antigen on RBCs. Compatibility testing showed five unrelated patients from Morocco and one female patient from Europe lacking this high-prevalence antigen. Whole-exome sequencing on the Moroccan samples identified the *SLC44A2* gene (also known as *CTL2*) on chromosome 19p13.2 as a primary candidate. Genomic DNA sequencing identified SNV c.1192C>A in all Moroccan samples, associated with amino acid change (p.Pro398Thr) on the third extracellular loop of the protein. L-929 cells transfected with the *SLC44A2* variant carrying c.1192A were non-reactive with the antibody, confirming the loss of this antigen associated with this amino acid change [12]. Conversely, overexpression of *SLC44A2* in L-929 cells demonstrated the antigen (Peyrard, personal communication, June 22, 2019).

The European patient exhibited a large deletion of the *SLC44A2* gene, including exons 1–14, resulting in a null phenotype. This was consistent with the serological data, which showed that while the sera of the Moroccan probands were compatible with RBCs from the European patient, the Moroccan RBCs were incompatible with the antibody from the European patient, indicating that the latter was an antibody directed against the *SLC44A2* protein [12].

There were additional clinically interesting features associated with this gene. *SLC44A2* knockout mice have hearing loss; the proband with null phenotype also has hearing loss in the upper-frequency range. The protein encoded by the *SLC44A2* gene also carries HNA-3 (Human Neutrophil Antigen), which is involved in transfusion-related acute lung injury; the possible association between the HNA-3a/3b status and red cell antigen expression remains unknown. To avoid confusion, the terminology used for the granulocyte antigen system HNA-3 and the blood group system CTL2, both encoded by *SLC44A2*, have not been synchronized but are being kept separate.

Thus, there are two RBC antigens within the CTL2 system (Table 1): one antigen, defined by the antibody in the European proband, is called VER (ISBT 039001) after Verona from the proband's birthplace. The other antigen is called RIF (ISBT 039002) after the geographical region in Morocco from which the patients who lack this antigen originate.

System 40: PEL

The PEL antigen was identified in 1980 and subsequently classified in the 901 series as 901014. PEL− RBCs have been found only in four unrelated French-Canadian families from the province of Quebec. Integrated genomic and proteomic studies were presented to explain this phenotype. Initially, whole-exome sequencing did not reveal any candidate sequence variants among family members. However, a comparative global proteomic analysis of the RBCs pinpointed ABCC4 as the likely candidate protein for PEL antigen expression. Review of exome sequencing data showed a structural change comprising a large deletion of the *ABCC4* gene in all PEL− cases. Sanger sequencing confirmed the same breakpoint. Subsequent western blot and transfection studies confirmed that the PEL blood group antigen is carried on the ABCC4 protein and that homozygosity for a large deletion in the *ABCC4* gene, chromosomal location 13q32.1, is responsible for the rare PEL− phenotype. The carrier protein, ABCC4, is a member of the superfamily of ATP-binding cassette transporters. The JR and LAN blood group systems (ISBT 032 and 033) are carried on by other members of this superfamily. ABCC4 protein is also called MRP4 for Multidrug Resistance Protein 4. The PEL− phenotype is associated with moderately impaired platelet aggregation [13].

PEL is now a new blood group system, ISBT 040, and the antigen transferred from the 901 series, where 901014 becomes obsolete. There is only one antigen known in this system: PEL or ISBT 040001 (Table 1). The name PEL is derived from the name of the first antigen-negative proband who made anti-PEL [14].

System 041: MAM

The MAM-negative phenotype was first described in 1993 in a pregnant woman (MAM.) who presented with an antibody to a high-prevalence antigen. Since then, severe or even fatal haemolytic disease of the fetus and newborn (HDFN) has been reported in such cases. The genetic basis for the MAM antigen remained unresolved, and the antigen was placed in the 901 series as 901016. Studies on samples gathered from 10 MAM-negative individuals were reported at the first virtual meeting. Genomic DNA from five MAM− subjects was subjected to whole-exome sequencing (analysis and a subsequent filtering strategy showed the only candidate gene was *EMP3* [encoding Epithelial Membrane Protein 3]); Sanger sequencing of *EMP3* confirmed variants were present in this gene for all 10 MAM− samples studied. A total of four different allelic variants were defined among the 10 MAM− samples: namely, a total gene deletion, two different single-exon deletions and a substitution that creates a stop codon. The latter is the most commonly encountered and was found in four of the 10 MAM− individuals. Its frequency in the genome aggregation database (gnomAD) is 0.017% (present in 43 of 251,000 alleles) [15].

Subsequent short hairpin (sh)RNA knockdown, CRISPR knock-out and transfection over-expression studies were used to show a causal relationship between *EMP3* and the presence or absence of the MAM antigen. The probands have no apparent clinical phenotype. *EMP3* has been reported to act both as an oncogene and a tumour suppressor gene in different cancers. The presented study showed a marked increase in reticulocyte production from MAM− erythroid cultures suggesting that *EMP3* acts as a suppressor of proliferation in normal erythropoiesis. It may also have a function in regulating the level and stabilization of CD44 at the cell surface of erythroid progenitors. As a consequence of this, it was shown that MAM− RBCs have consistently low expression of CD44, which affects red cell levels of the antigens in the IN system (ISBT 023) [15].

The MAM antigen was transferred from the 901 series into the MAM blood group system, ISBT 041, where 901016 became obsolete. There is only one known antigen in this system: MAM or ISBT 041001 (Table 1).

System 042: EMM

The new blood group system EMM was reported independently by two teams at the second virtual meeting [16, 17].

Antibodies to a high-prevalence antigen, Emm, were first described in 1987 [18]. One team investigated samples from a 65-year-old Indian male who had presented with anti-Emm, as well as samples from family members. Whole-genome sequencing and variant filtering defined a homozygous 2 bp deletion, c.2624_2625delTA, in the *PIGG* gene for the proband [17]. The heterozygous 2 bp deletion was also observed in two other family members. *PIGG* encodes a glycosylphosphatidyliniositol (GPI) ethanolamine phosphate transferase-2 enzyme. This enzyme is involved in the addition of a side chain modification on the second mannose of a GPI protein anchor in the red cell membrane. Further studies

on a frozen sample collection of historic anti-Emm probands from Japan, United States and North Africa, revealed three further loss-of-function mutations in the *PIGG* gene: deletion of exons 2–3, deletion of exons 7–9, insertion–deletion in exon 3 [17]. There are reports of neurological phenotypes, including seizures and developmental delay involving *PIGG* loss of function mutations [19].

An independent team studied samples from three unrelated Emm− individuals, all with anti-Emm. Samples were obtained from cryopreserved reference material. Whole-exome sequencing and data analysis defined three different predicted loss-of-function mutations in the *PIGG* gene three different predicted loss-of-function mutations in the *PIGG* gene: c.640C>T (p.His214Tyr), c.901+1delG, deletion of exon 6. Flow cytometry studies revealed a strong decrease in Emm expression in *PIGG* knockout cells but normal expression of CD59. The CD59 blood group antigen is expressed on the protein portion of the GPI-linked protein. K562 cells deficient of *PIGA* lacked both Emm and CD59, as the GPI was completely absent [16].

In summary, two different approaches demonstrated that the *PIGG* gene, located on chromosome 13, encodes the Emm antigen in this new system, EMM ISBT 042 (Table 1) [16, 17]. The Emm antigen was transferred from the 901 series, making 901008 now obsolete and is defined as ISBT 042001, the only antigen in this system. The name comes from the first antigen-negative proband to make anti-Emm.

System 043: ABCC1

An antibody to an unknown high-prevalence red cell antigen was detected in a young Brazilian man who had been transfused when undergoing a kidney transplant [20]. Parents of the proband with the antibody were first-degree cousins. Samples were available for study from the proband and three siblings, one of them being compatible with the proband. Whole-exome sequencing, with variant filtering strategy, defined a homozygous large deletion encompassing five exons in the *ABCC1* gene in the proband and the compatible sibling. The other family members were heterozygous. Sanger sequencing was used to define the breakpoints of the deletion. Expression studies in K562 transfected cells showed the antibody to react with the *ABCC1* gene product expressed on the transfected cells. Allele-specific PCR confirmed that the parents were each heterozygous for the deletion.

ABCC1 is reported to play a role in the protection of kidney epithelial cells. Regrettably, the proband died from renal failure further to kidney graft rejection. The proband's brother showed evidence of abundant oxalate crystals in urine, which is a potential early marker for renal disease. The carrier protein ABCC1, a member of the superfamily of ATP-binding cassette transporters, has practical clinical relevance in pharmacogenomics and pharmacokinetics [21]. Other ABC transporters are also known to carry antigens of blood group systems, namely *ABCG2* of the JR system (ISBT 032), *ABCB6* of the LAN system (ISBT 033) and the above *ABCC4* of the PEL system (ISBT 040) [1, 13, 22–24]. ABCC1 was proposed as the carrier of a new blood group system designated ISBT 043, encoded by *ABCC1*. This is the first blood group system encoded by a gene on chromosome 16 (Table 1).

The wild-type allele *ABCC1*01* encodes the high-prevalence WLF antigen after the proband's name, assigned as ISBT 043001. Homozygosity for the null allele, *ABCC1*01N.01*, with the exon deletion, defines the WLF− phenotype.

NEW BLOOD GROUP ANTIGENS IN EXISTING BLOOD GROUP SYSTEMS

Fifteen blood group antigens were added to existing blood group systems over these meetings. These included high- and low-prevalence antigens (Table 2). Of note, one, the Kg antigen, was previously in the 700 series and shown to be on the Rh-associated glycoprotein system (RHAG ISBT 030). The remainder represented de novo discoveries.

System 002: MNS, SUMI (ISBT 002050)

A positive cross-match in a Japanese patient revealed an antibody to an unknown low-prevalence antigen, tentatively named SUMI [25]. Monoclonal anti-SUMI-producing cells (HIRO-305) were established using lymphocytes from a donor having anti-SUMI. SUMI+ RBCs were examined by immunocomplex capture fluorescence analysis, identifying glycophorin A as a carrier candidate of the antigen. Genomic DNA was extracted from whole blood, and the *GYPA* gene of donors with SUMI+ RBCs was shown to carry a c.91A>C (p.Thr31Pro) substitution. The serologic screening revealed that 23 of 541,522 Japanese individuals (0.0042%) were SUMI+, whereas 1351 of 10,392 investigated individuals (13.0%) had alloanti-SUMI. SUMI antigen is sensitive to ficin, trypsin, pronase and neuraminidase, slightly weakened by α-chymotrypsin and resistant to sulfhydryl-reducing agents. Based on their prevalence and chemical sensitivity, anti-SUMI has been suggested to be a naturally occurring IgM. There are now 50 antigens in the MNS system, including SUMI, defined numerically as ISBT 002050.

System 004: Rh, CETW (ISBT 002063)

A genomic study on indigenous Australian individuals revealed two individuals (2/247) heterozygous for an *RHCE* variation c.486C>G. This novel SNV, c.486C>G on the *RHCE* allele predicts an amino acid change, p.Asn162Lys in the third extracellular domain of the RhCE blood group protein. These findings triggered the review of an unresolved HDFN case involving a woman with an unidentified antibody directed to a low- prevalence antigen present on the paternal and newborn's RBCs. Sequencing showed both the father and the newborn to be heterozygous for the same c.486C>G SNV in *RHCE*.

Extensive serologic testing on the stored maternal plasma and the antibody eluted from the newborn's RBCs showed the maternal

TABLE 2 New antigens added to pre-existing blood group systems by the Working Party: Reported at the business meeting in Basel in June 2019, the second virtual business meeting in December 2020 and the third virtual business meeting in June 2021

Business meeting	ISBT number	Blood group system	Antigen number	Alt. name	Antigen prevalence	Allele name	Molecular basis	Protein change	SNV (rs-number)	GenBank	Reference
Second virtual	ISBT 002	MNS	MNS50	SUMI	Low	*GYPA*50*	*GYPA* *c.91A>C*	GPA p.Thr31Pro	Pending	LC495310	[25]
Third virtual	ISBT 004	RH	RH63	CETW	Low	To be determined	*RHCE* *c.486C>G*	RhCE p.Asn162Lys	rs199725473	MZ326705	Manuscript in preparation
Basel	ISBT 005	LU	LU28	LUNU	High	*LU*02.-28*	*BCAM* *c.121G>A*	BCAM p.Val41Met	rs957795435	MK965667	[26]
Basel	ISBT 005	LU	LU29	LURA	High	*LU*02.-29*	*BCAM* *c.1351A>C*	BCAM p.Lys451Gln	rs28399630	Pending	[27]
Third virtual	ISBT 010	DI	DI23	DIST	Low	*DI*02.23*	*SLC4A1* *c.1447G>A*	CD233 p.Gly483Ser	rs544557335	Pending	[28]
Second virtual	ISBT 013	SC	SC8	SCAR	High	*SC*01.-08*	*ERMAP* *c.424C>G*	ERMAP p.Gln142Glu	Pending	MK933825	[29]
Second virtual	ISBT 013	SC	SC9	SCAC	High	*SC*01.-09*	*ERMAP* *c.217C>T,* *c.219C>T*	ERMAP p.Arg73Cys p.Arg73Cys	rs149787850 rs33954154	MW427218	[31]
Second virtual	ISBT 020	GE	GE12	GECT	High	*GE*01.-13*	*GYPC* *c.59C>T*	GPC p.Pro20Leu	rs143216051	Pending	[32]
Second virtual	ISBT 020	GE	GE13	GEAR	High	*GE*01.-14*	*GYPC* *c.118G>A c.333A>C*	GPC p.Gly40Arg p.Gly111Gly	rs772372126 rs1050967	Pending	[33]
Basel	ISBT 022	KN	KN10	KDAS	Average	*KN*01.10*	*CR1* *c.4843A>G*	CR1 p.Ile1615Val	rs6691117	Pending	[35]
Second virtual	ISBT 022	KN	KN11	DACY	Average	*KN*01*	*CR1* *c.3623A*	CR1 p.His1208	rs2274567	Pending	[36]
Second virtual	ISBT 022	KN	KN12	YCAD	Average	*KN*01.12*	*CR1* *c.3623A>G*	CR1 p.His1208Arg	rs 2274567	Pending	[36]
Basel	ISBT 026	JMH	JMH7	JMHN	High	*JMH*01.-07*	*SEMA7A* *c.709G>A* *c.1545A>G* *c.1865G>A*	CD108 p.Asp237Asn p.Gln515Gln p.Arg622His	rs140707085 rs741761 rs140128092	Pending	[37]
Second virtual	ISBT 026	JMH	JMH8	JMHA	High	*JMH*01.-08*	*SEMA7A* *c.507C>T* *c.556G>A* *c.1545A>G*	CD108 p.Tyr169Tyr p.Glu186Lys p.Gln515Gln	rs2075589 rs572867366 rs741761	MT017654	[39]
Second virtual	ISBT 030	RHAG	RHAG5	Kg	Low	*RHAG*01.-03*	*RHAG* *c.490A>C*	RhAG p.Lys164Gln	rs144305805	LC508243	[41]

Abbreviations: GPA, glycophorin A; GPC, glycophorin C; SNV, single nucleotide variant.

antibody reacted with the RBCs from the two indigenous Australians but not with control cells. The combined studies indicate that the c.486C>G SNV generated a novel low-prevalence antigen. The antigen name is 'CETW' with 'TW' representative of the town for the Indigenous Australian study. While there are now 56 antigens defined in the RH system, the ISBT designation of CETW is ISBT 004063, noting that seven antigens have been made obsolete.

System 005: Lutheran LUNU (ISBT 005028) and LURA (ISBT 005029)

Two high-prevalence antigens were added to the Lutheran, LU, blood group system (Table 2). An antenatal patient of Caucasian descent exhibited an unidentified antibody that was reactive with all tested RBCs except examples of In(Lu) cells. Soluble recombinant Lu protein inhibited the antibody. Other known LU high-prevalence antigens were ruled out. Sanger sequencing of all 15 exons of the *BCAM* coding region, which encodes the Lu glycoprotein, identified homozygosity for an SNV, c.121G>A (p.Val41Met). This c.121G>A is listed in the gnomAD database with a frequency of 3.98×10^{-6}. Family studies demonstrated inheritance with the parents and two siblings heterozygous for this allele [26]. This antigen is named LUNU (LU 028), LU for Lutheran and NU from the name of the patient.

The second novel antigen was detected from studies involving an individual of Arab descent with beta-thalassemia intermedia and alpha globin deletion who showed an antibody to a high-prevalence antigen in the LU system. Soluble recombinant Lu protein inhibited the antibody. Sequencing the *BCAM* coding region identified c.1351A>C (p.Lys451Gln) as the only variant in the gene [27]. This antigen is named LURA (LU 029), LU for Lutheran and RA from the name of the patient.

System 010: Diego, DIST (ISBT 010023)

A novel low-prevalence Diego antigen named DIST was characterized by a c.1447G>A SNV in *SLC4A1*, leading to a p.Gly483Ser amino acid change in the extracellular part of the Band 3 protein [28]. This variant was on the *DI*B* allele (phenotype was Di(a-b+)). According to the gnomAD database, this variant is found in one of 113,680 European individuals. The clinical significance of the antibody is unknown. The name of the antigen 'DIST' results from DI for Diego and the first letters of the first names of the person producing the antibody and the antigen carrier, respectively.

The new DIST (DI 010 023) antigen is defined by c.1447G>A (p.Gly483Ser), with a total of 23 antigens now defined in this system.

System 013: Scianna, SCAR (ISBT 013008) and SCAC (ISBT 013009)

Two new antigens, designated SCAR and SCAC, were added to the Scianna, SC system.

The SCAR antigen was identified following the detection of an antibody to a high-prevalence antigen in a patient from Saudi Arabia with sickle cell beta thalassemia [29]. For investigating the antibody specificity, soluble recombinant ERMAP protein was used, which inhibited the antibody in the column agglutination technique. The patient showed no signs of haemolysis following an incompatible blood transfusion. A monocyte monolayer assay (MMA) was negative, indicating a possible low level of clinical significance for this antibody [29].

The genomic sequence of *ERMAP* showed the patient exhibited homozygosity for an *ERMAP* c.424C>G (p.Gln142Glu) variant, for which his two sisters were heterozygous. The c.424C>G variant occurred in the *SC*01* allele. The frequency of the variant was not known since this variant was not reported on any database. The name 'SCAR' for the new antigen was derived from SC for the blood group system and AR from the patient's name. Individuals homozygous for this c.424G allele do not express the high-prevalence SCAR antigen and can produce anti-SCAR. The allele, *SC*01.-08*, occurs in one haplotype with the *RHCE*03* (*RHCE*cE*) allele. The new SCAR (SC 013008) antigen is the eighth antigen of the SC blood group system and the first new SC antigen reported since 2005 [30].

Another new SC antigen, SCAC, was discovered following the identification of an antibody to a high-prevalence antigen in the plasma of a 41-year-old African American female with sickle cell disease (SCD) undergoing kidney biopsy [31]. A compatible sister was found. An MMA was performed and suggested the clinical significance of the antibody. The antibody reacted with ficin, papain, trypsin, α-chymotrypsin and 0.2 M DTT-treated cells. The only non-reactive cells were SC_{null} cells, indicating a probable Scianna-related antibody.

Sequencing of *ERMAP* coding exons found the sample was homozygous for exon 4 changes, c.217C>T (p.Arg73Cys) and c.219C>T (silent), with frequencies of 0.00811 and 0.0838, respectively, in Africans. The c.217T (p.Cys73) change in *ERMAP* suggested the patient had an altered protein, encoded by *SC*01.-09*, identifying a new SC antigen, ISBT 013009, named 'SCAC' after SC for Scianna and AC for the amino acid changes (Arg to Cys).

Analogous to the SCAR antigen, here homozygosity for the *SC*01.-09* allele abolishes expression of SCAC on the ERMAP protein. SCAC is the ninth antigen in the SC system (Table 2).

System 020: Gerbich, GECT (ISBT 020013) and GEAR (ISBT 020014)

Two new Gerbich, GE, high-prevalence antigens, GECT and GEAR, bring the total number of antigens in this system to 13, noting that one was previously made obsolete.

A sample came from a 13-year-old girl with SCD, recently transfused, who experienced a delayed haemolytic transfusion reaction [32]. The antibody reacted with all panel cells except those with the phenotype GE:−2,3,4 and GE:−2,−3,4. *GYPC* Sanger sequencing revealed homozygosity for a novel SNV, c.59C>T (predicting p.Pro20Leu). The patient's brother, also with SCD, had the same *GE*

genotype, but RBCs were not available to test for compatibility. It was concluded that homozygosity of the novel *GYPC*, c.59T (p.Leu20) resulted in the loss of glycophorin C (GPC) epitopes, for example, negativity for the antigen 'GECT'. The *GYPC* c.59C>T has a frequency of 0.01246 in Africans. The proposed antigen name is GECT with GE for Gerbich and CT after the C to T nucleotide exchange. The allele was named *GE*01.-13*.

In a second case, a sample was received from a 38-year-old Caucasian woman with a possible delayed transfusion reaction, whose plasma contained an antibody to a high-prevalence antigen [33]. Phenotypically similar RBCs treated with 0.2 M DTT or α-chymotrypsin were reactive, but the plasma was non-reactive with papain or trypsin-treated RBCs, suggesting possible Gerbich specificity. An eluate containing the antibody was made by adsorption/elution and reactivity patterns suggested anti-Ge3 specificity. However, the patient's RBCs typed Ge2+ and Ge3+ using single donor source antibodies and Ge4+ with monoclonal anti-Ge4. *GYPC* exon sequencing identified a novel homozygous nucleotide change in exon 3, c.118G>A (p.Gly40Arg). This change is present in the dbSNP database with a rare minor allele frequency (MAF) of 0.0000247. A synonymous change c.333A>C in exon 4 was also found. Data were consistent with a novel high-prevalence antigen in the Gerbich system that was named 'GEAR'. Lack of the antigen is predicted to be associated with the homozygous presence of c.118G>A SNV in *GYPC* exon 3 encoding amino acid change p.Gly40Arg in GPC (or p.Gly19Arg in GPD). The role of anti-GEAR as causative in the patient's delayed transfusion reaction is unknown due to the presence of other antibodies. The allele name of the *GYPC* exon 3 c.118G>A SNV is *GE*01.-14*.

GECT (GE 020013) and GEAR (GE 020014), both of high prevalence, were added to the GE blood group system.

System 022: Knops, KDAS, DACY and YCAD (ISBT 022010, 022011 and 022012)

Three new antigens complete two new antithetic relationships of the Knops (KN) blood group system, KCAM/KDAS and DACY/YCAD, respectively [1, 34–36].

One new antithetic relationship was defined by investigating a Caucasian male patient who had been transfused and who presented with an antibody to a low-prevalence antigen. The antibody reacted with the RBCs of a donor included on an antibody identification panel that lacked the high-prevalence Knops antigen, KCAM. An antigen antithetical to KCAM has not previously been defined. The *CR1* gene encodes the glycoprotein that carries the Knops antigens, and loss of the KCAM antigen is known to arise from *CR1* c.4843A>G change, which results in p.Ile1615Val. Additional testing showed this patient's antibody to be non-reactive with KCAM+ cells from 23 individuals homozygous *CR1* c.4843A but reactive with KCAM+ cells from individuals heterozygous *CR1 c.4843A/G* and with KCAM− cells from individuals homozygous *CR1* c.4843G [35].

KCAM is defined by p.Ile1615, and the antithetical antigen to KCAM was named KDAS and is defined by the amino acid change from isoleucine (KCAM) to valine (KDAS). The antigen name KDAS (ISBT 022010) was derived from the name of the proband. The allele for KDAS will be defined as *KN*01.10*.

The second new antithetic relationship within the Knops blood group system is represented by two new KN antigens with unusual, previously unreported locations on the CR1 protein. To date, all previously described antigens of the KN blood group system are located in the long homologous repeat D (LHR-D) of complement receptor 1 (CR1). While there have been reports that some sera react only with the LHR C (LHR-C), the antigens in LHR-C were unknown. Soluble recombinant LHR-C and LHR-D were used to identify antibodies directed to LHR-C of CR1, into which a point mutation was introduced to characterize the underlying blood group antigens [36]. In addition, database studies to define haplotypes of *CR1* were performed. Several antisera were identified that were specific, either against CR1 p.His1208 or against CR1 p.Arg1208, a polymorphism described by rs2274567 and located in LHR-C. It was shown that due to linkage disequilibrium, anti-CR1 p.His1208 might be mistaken for anti-KCAM. In summary, a novel antithetical KN blood group antigen pair was found at position p.1208 of CR1. Antibodies against these two novel antigens seem to contribute to more than a quarter of all KN antibodies in Europe. The names DACY (dual antigen-site of CR1 beyond LHR-D) for CR1 p.His1208 and the reverse name YCAD for CR1 p.Arg1208 were proposed. With these additions, there are now 12 antigens in the KN system.

All four permutations, that is, KCAM encoded together with DACY or YCAD on the same allele, and KDAS encoded together with DACY or YCAD on the same allele, were observed [36]. This permuted polymorphism is found with varying prevalence among the different ethnic groups studied. Moreover, most of these permutations are linked to already recognized *KN* alleles, splitting these latter into up to four different allelic subtypes.

System 026: JMH, JMHN (ISBT 026007) and JMHA (ISBT 026008)

A further antigen was added to the JMH system at the Basel meeting. An antibody to a high-prevalence antigen was identified in a Moroccan individual and shown to be inhibited by soluble recombinant JMH (Table 2). Genomic sequencing revealed homozygosity in the *SEMA7A* gene for three SNVs. A rare non-synonymous variant, c.709G>A (p.Asp237Asn); a common synonymous change, c.1545A>G (p.Gln515Gln) and a rare non-synonymous c.1865G>A (p.Arg622His). Notably, all antigens in JMH are changes from arginine. Modelling shows that p.Arg622 is exposed out of the RBC membrane, and this may be a critical amino acid [37]. It is noted that this variant has a MAF of 0.002 in Europeans and 0.0008 in Africans in the database that was established by the 1000 Genomes Project Consortium (1KGP), a construct of genome sequences for 2504 individuals [38]. This is the seventh antigen of

the JMH blood group system (ISBT 026007), and the proposed allele name is *JMH*01.-07*. The new antigen was designated JMHN, where N stands for North Africa, where all cases of the rare JMH:−7 type have been found to date.

Another novel JMH antigen, JMHA, was described following the serological investigation of plasma from a female Palestinian patient that showed the presence of an antibody to a high-prevalence antigen [39]. Soluble recombinant JMH blood group proteins were used in inhibition tests. The clinical significance of the antibody was assessed by an MMA and showed a monocyte index <3%, suggesting that the antibody is of minor or negligible clinical relevance. The plasma of the patient was crossmatched with JMH:−3, JMH:−5 and JMH:−7 cells, all found to be incompatible, whereas JMH:−1 cells were found to be compatible. Sequencing of *SEMA7A* revealed a homozygous substitution, c.556G>A (p.Glu186Lys, rs572867366) and two silent polymorphisms c.507C>T (p.Tyr169Tyr, rs2075589) and c.1545A>G (p.Gln515Gln, rs741761). Whereas the c.556G>A variant is only present in 2% of the Esan population (Nigeria) dataset of the 1KGP databank and was not observed in other populations, the variants giving rise to the two silent mutations were commonly observed, showing respective frequencies of 0.22 and 0.495 averaged over all populations.

The new antigen was designated the eighth antigen of the JMH blood group system (ISBT 026008) and called JMHA, with 'A' standing for 'Arabia' as the patient was from Qatar. The proposed allele name is *JMH*01.-08*.

System 030: RHAG, Kg (ISBT 030005)

The Kg antigen was first discovered in an investigation of a woman whose newborn had HDFN [40]. The molecular nature of the Kg antigen has remained a mystery for over 30 years. A monoclonal antibody to the Kg antigen and recombinant protein were developed that allowed for immunoprecipitation analysis [41]. Immunoprecipitates from the proposita's RBC ghosts were subjected to mass spectrometry analysis. Carrier candidates of the antigen were *RHD*, *RHCE* and *RHAG*. Whereas *RHD* and *RHCE* were normal, DNA sequence analysis showed a heterozygous SNV in the third exon of *RHAG*, with c.490A>C (p.Lys164Gln) in a Kg-positive sample. The candidate for the Kg antigen was molecularly isolated and confirmed to be a determinant of the Kg antigen by cell transfection and flow cytometry analyses. The Kg antigen and the genetic mutation were then screened for in 61,362 Japanese blood donors. Kg phenotyping further clarified that 0.22% of the Japanese population studied was positive for the Kg antigen.

In 2010, an *RHAG* c.490A>C homozygous individual of Japanese origin with an antibody against the high-prevalence antigen DSLK was reported [42]. Kg appears to be antithetical to DSLK; however, this has not been confirmed by cross-testing with anti-DSLK since this antibody is no longer available. Kg has been transferred from the 700 series antigen, previously 700045 'Katagiri' (Kg), into the RHAG system as RHAG5.

CHANGES TO BLOOD GROUP ALLELE TABLES

There were changes made to the names of blood group alleles in the VEL, RAPH, RHAG and CROM systems where a minus sign had previously occurred after the star sign. For example, the *VEL*-01* allele was renamed to *VEL*01N.01*, which is the predominant null allele. The *CROM*-01* was renamed to *CROM*01.-01*, which defines an allele differing from the reference allele by a variant that results in the loss of one of the high-prevalence antigens expressed by the reference and for which the antithetical low-prevalence antigen has not been reported. The allele for KDAS will be defined as *KN*01.10*, which makes *KN*01.-09* obsolete (this latter indicated the loss of an antigen before the discovery of the antithetical antigen).

Finally, an overview of all currently known antigens can be found on the ISBT webpage (https://www.isbtweb.org/fileadmin/user_upload/Table_of_blood_group_antigens_within_systems_v10.0_30-JUN-2021.pdf). This list is supplemented by a table of 'obsolete antigens', which is also available on the WPs website.

CONCLUSION

This report described the registration of new blood group systems and new antigens into existing systems over the course of four business meetings. The ISBT now recognizes 378 antigens, of which 345 are clustered within 43 blood group systems. The remaining 33 still have an unknown genetic basis and remain classified in either the 200 collections or the 700 or 901 series, awaiting the resolution of their genetic background.

It is of note that advances in genomic, proteomic and cellular technologies are contributing to this current wave of discoveries. This is contributing to both de novo discoveries and resolving antigens previously listed in the ISBT 200, 700 and 901 series. In addition, genomics continues to reveal a further sub-layer of blood group alleles associated with altered blood group phenotypes.

The ongoing recognition of new blood group systems and antigens underscores the diverse and complex biology of the red cell membrane. The curation of blood group systems, antigens and associated alleles is of clinical significance to provide for accurate typing and reporting for both donors and patients in the transfusion and antenatal settings. The Working Party continues to update the blood group antigen tables and the allele nomenclature tables. These can be found on the ISBT website (http://www.isbtweb.org/working-parties/red-cell-immunogenetics-and-blood-group-terminology/).

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C.A.H. and C.G. wrote the manuscript. All authors critically reviewed, revised and commented on the manuscript. As members of the Working Party, all authors have contributed equally to the discussion and conclusions drawn in this paper. All authors approved the final version of the paper.

CONFLICT OF INTEREST

C.G. acts as a consultant to inno-train Diagnostik GmbH, Kronberg i. T., Germany. Procedures for the molecular detection of *GYPB* deletions for S-s-U- phenotype diagnostics have been granted as a European patent (EP 3 545 102 B1). A similar content patent US application is pending. G.A.D. is a transfusion education consultant for Abbott Laboratories, Chicago, IL. W.J.L. is a member of the Scientific Advisory Board of CareDx, Inc. and his institution is a founding member of the Blood Transfusion Genomics Consortium (BGC) that has received fees from Thermo Fisher Scientific Inc. to help co-develop a high-density DNA genotyping array. C.M.W. is a member of the Scientific Advisory Board of Quotient. N.G., M.L.O. and J.R.S. have no conflicts of interest with regard to this manuscript. L.C., Q.C., F.B.C., W.A.F., A.H., J.Y., M.A.K., P.L., C.L.F., N.N., T.P., Y.T., N.T., E.V.D.S., B.V., F.F.W., C.W., S.W., V.Y. and C.H. have no conflicts of interest.

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APPENDIX

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