RhD detection in transfusion medicine

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Abstract

Transfusion medicine represents one of the emerging medicine branches of the last century. With the discovery of isoagglutinin, the main proof has been made that blood group inheritance is encoded in genes which established the theory of four blood groups. Moreover, the presence of certain antibodies, later known as Rh factors, directly reflects the ability to accept or reject of blood group. This review will cover the recent information about blood group and the method for detection, focusing on molecular methods. Several methods of detection of Rh factor have been used in transfusion medicine which helps in diagnostic procedures. This is of great importance, especially during pregnancies or during blood donation processes. Molecular approaches enable the identification and analysis of blood that is negative for specific antigens against which a patient may have generated antibodies, the detection and analysis of antibodies, and the identification of donors who are negative for common antigens.

Keywords: RhD antigen, blood type, inheritance, variants, antiglobulin

1. Introduction

Transfusion medicine includes voluntary blood donation, collection of blood products, detection of coagulation disorders, blood testing for infectious diseases, tissue typing, immunohistological testing, and control of the use of blood products. The first serious steps in transfusion medicine can be found in the 20th century when Karl Landsteiner published a text on the "Agglutination Phenomena of Normal Human Blood" in the Wiener Klinische Wochenschrift in 1901 [1]. Landsteiner stated that not every blood group fits every person and that mixing the blood of two people could sometimes lead to problems. He discovered isoagglutinin in human blood and proved the existence of four blood groups (A, B, AB, and 0). His work opened wide horizons in all branches of medicine, and the proof that blood groups are inherited by certain genes became the basis of the development of genetics [1,2]. The definition of the blood groups is that they are hereditary characteristics of blood cells including erythrocytes, leukocytes, platelets, and plasma proteins, and that they are coded by genes inherited from parents. Most attention is given to erythrocyte blood groups. More than 400 erythrocyte blood group characteristics -antigens - have been proven on the erythrocyte membrane. According to their similarity, they are classified into more than 20 blood group systems. Some of them are ABO, Rh, Kell, Duffy, and Kidd [2].
A person is inherently hypersensitive (has natural antibodies) to a foreign blood group in the ABO system of blood groups. A person develops hypersensitivity to another person's blood group the quickest in the Rh system. Inappropriate blood transfusion might result in the recipient losing their life [3].
Humans have blood plasma that contains antiA or antiB antibodies to these proteins. These antibodies are triggered and treat other people's blood as an invader if "wrong" blood enters the bloodstream. The mismatched blood type donation can result in potentially fatal problems [3,4]. The body initiates an immunological response and rejects the transfusion if someone with group B antigen receives red blood cells from someone with group A antigen [4].

Blood clots may develop as a result of the recipient's plasma attacking and destroying the donor's cells, which can obstruct blood vessels. Anaphylaxis and allergic responses are additional potential adverse effects.

A person with an Rh+ blood group can receive blood from someone with an Rh- factor, but the reverse is not possible. A person who has the Rh- factor can only get Rh- blood. Rh positive person can receive from Rh+ and Rh-. According to research, 0 negative is a universal donor, while AB positive can receive from anyone. D antigen is found in RhD-positive people, who make up around 85% of the population overall, but not in RhD-negative people [5].

It is rare to find antibodies from this system in people who have not received a transfusion or in women who have not given birth. These antibodies are formed by interaction with foreign erythrocyte antigens. Antibodies in the immune system are antibodies that they emerge when an individual was transfused with erythrocytes containing antigens that she does not have, or the pregnant lady is immunized with the child's erythrocytes that entered her circulatory system, and the child acquired those antigens from the father [5].

There are currently 55 antigens known to belong to the Rh system, however, C, c, D, and E are the most significant [5]. The Rh factor is used to evaluate if the D antigen is present or not. The patient is Rh D (+) if antigen D is present on the erythrocyte membrane. The patient is Rh D (-) if antigen D is not present. Antigen D has the highest immunogenicity among all of the Rh system antigens. Typical allo-immune antibodies from the Rh system are created as a result of synthesis prompted by erythrocyte transfusion or pregnancy. The most prevalent of all the antibodies produced by the Rh system is anti-D, which can form in pregnant RhD-negative women and is responsible for infant hemolytic illness [7].

Red blood cells that have antigen A on their surface correspond to blood group A. In this case, antibodies against blood group B are present in the liquid part of the blood, or plasma.

Red blood cells that have antigen B on their surface correspond to blood group B. Then, antibodies against blood group A are present in the plasma. If neither of these two antigens is found on the outer surface of the red blood cells, it corresponds to blood group 0. Antibodies against blood group A and blood group B are present in the plasma then. It is also possible that both antigen A and antigen B are present on the surface of red blood cells, and then there are no antibodies in the plasma, and that case corresponds to blood group AB [8].
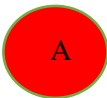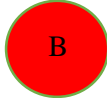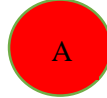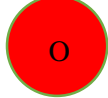
	GROUP A	GROUP B	GROUP AB	GROUP 0
Red blood cell type	A	B	A	O
Antibodies in plasma	Anti-B	Anti-A	None	Anti-A and Anti-B
Antigens in red blood cell	A antigen	B antigen	A and B antigens	None

Figure 1: The figure illustrates the variations in blood types and associated antigens [8].

1.1. Inheritance of blood groups

By joining the father's and mother's gametes, the child inherits one gene from each parent for the AB0 blood group system and the Rh factor. Genes A and B are codominant (equal), and gene 0 is recessive (subordinate) [9]. This means that a child with genome AA and A0 has blood group A. A child with genome BB and B0 will be in blood group B. To have blood group 0, he must inherit gene 0 from his father and mother. The gene for the D antigen of the Rh system can be expressed or muted.

If a child inherits an expressed gene from at least one parent, he will have the D antigen on his erythrocytes, that is, the Rh+ phenotype [9].

Table 1. Inheritance combination

The blood group of parents	The possible blood group of baby	Impossible
A and A	A, O	B, AB
A and B	A, B, AB, O	none
A and AB	A, B, AB	O
A and O	A, O	B, AB
B and B	B, O	A, AB
B and AB	A, B, AB	O
B and O	B, O	A, AB
AB and AB	A, B, AB	O
AB and O	A, B	AB, O
O and O	O	A, B, AB

As can be seen from Table 1, each person inherits two AB0 genes, one from each parent. Genes A and B are dominant, and the 0 gene is recessive. Persons who inherited genes A and 0 from their parents, respectively. genes B and 0, are called heterozygotes, while people who have inherited two A or two B genes are homozygotes. Both of them have antigen A on their erythrocytes, respectively. B. For example, parents heterozygous for gene A, father A0, and mother A0, can have children of blood group A or 0. In the case of heterozygous parents A0 and B0, children can have blood groups A, B, AB, and O. In the combination of AA and BB, children can be A, B, or AB, but never 0 blood group [9].

Why pregnant women with Rh- need to take medications throughout pregnancy is one of the most crucial points that need to be underlined. Less fetal blood enters the mother's system during childbirth. The erythrocytes that enter the mother's circulation, if the mother is Rh- and the kid is Rh+, will result in the creation of anti-RhD antibodies of the IgG group, which are antibodies to the D antigen and are permanently present in the mother's blood. These antibodies cross the placenta during the subsequent pregnancy and assault the fetus's erythrocytes if the infant is Rh+. They cause hemolysis, anemia, and tissue damage to the fetus, and in severe cases, death. The disease is known as mentioned above- a hemolytic disease of the newborn [10].

Pregnant women have their blood group and Rh factor determined at the beginning of pregnancy, and the titer of anti-Rh antibodies is monitored during pregnancy.
Rh-negative mothers after each delivery or termination of pregnancy should receive a drug containing therapeutic anti-RhD antibodies that quickly destroy erythrocytes remaining in the mother's blood. Thus, the mother's immune system does not have time to develop antibodies that would damage the child in the next pregnancy [11].
A kid can also develop hemolytic illness from antigens from other blood group systems (Duffy, Kell) less frequently, usually when there is no incompatibility between the Rh and AB0 systems. Antibodies of the AB0 system found in the mother's blood are of the IgM class and do not cross the placenta, so they do not cause a transfusion reaction in the newborn [12].

1.2. Inheritance of the Rh system

Rh antigens are genes that have two nearby loci (D and CcEe). The RhD gene, or gene D, is located on the first locus. One of the four alleles (ce, Ce, cE, and CE) and the RhCE gene, are located at the second locus. The Mendelian model of gene inheritance with two alleles governs the inheritance of these genes. Rh genes are a group of several alleles because immunological tests can identify numerous subtypes of the two basic types of alleles [13].
Rh genes are passed down in the form of complexes made up of two distinct haplotypes, one from each parent. A haplotype can be described as a set of all alleles of different loci of one chromosome, which are responsible for the expression of the observed property [13], in this case, the presence or absence of Rh (D) antigen. Rh genes are codominant and the antigens they determine are found on the erythrocyte membrane.
Therefore, antigens C and/or c, or E and/or e, are always found on erythrocytes. D-antigen has no counterpart. For the sake of a simplified understanding of inheritance, this missing antigen is referred to only as d in the professional literature. Rh (D) positive person- that person always inherited one or two Rh (D) genes from her

parents. An Rh (D) negative person does not have any Rh (D) gene and thus does not have Rh (D) antigen on erythrocytes [14].

Table 2: Representation of Rh haplotypes [15].

Weiner	Fisher-Race	Antigens present
R0	Dce	D, c, e
R1	DCe	D , C , e
R2	DcE	D , c , E
RZ	DCE	D , C , E
r	dce	c,e
r'	dCe	C, e
r"	dcE	c, E
r^y	dCE	C, E

Various notations describe the various possible haplotypes that can be created using the D, C, c, E, and e antigens. The frequently used Fisher-Race and Weiner nomenclatures are listed in the table below. In the Weiner notation, a capital "R" stands for the expression of RhD antigen and a lowercase "r" for its lack. The subscript 0 denotes the presence of the ce antigens in trans, 1 denotes Ce, 2 denotes cE, and Z denotes CE among RhD-positive patients ("R"). A single apostrophe denotes Ce, a double apostrophe denotes cE, and the letter y denotes CE among RhD-negative patients ("r"). The absence of any subscript shows the ce antigens in cis. Because each haplotype in the previous table is inherited from a single parent, two occurrences are needed to represent a person's whole Rh phenotype. R1R1, for instance, denotes homozygosity and the patient expresses just the D, C, and e antigens, each with a double "dosage" (i.e., strong antigen expression). The antigens D, C, c, and e are expressed, as shown by the code R1r. D, C, and c are expressed here using a single "dose," while e is expressed using two "doses"[15].

1.3. D antigen

The D antigen erythrocyte membrane, which is a result of RHD gene deletion, is present in more than 85% of Europeans, while 14 % do not have it [16]. Gene cloning and sequencing were used to identify the Rh system's basic underpinnings. Several genetic pathways, including the previously stated gene deletions, insertions, conversions, and mutations, have been described as causing D antigen variation [16]. Wagner et al. looked into the cellular causes of D-variants. Due to their origin in duplication, the RH genes (RHD, RHCE) on the first chromosome, which code for the antigens D and CcEe, are extremely homologous, whereas the gene for RhAG on the sixth chromosome is only 40% homologous to the RH genes. Each of the RH and RHAG genes has ten exons [16].

Weak D-variants are primarily caused by point mutations of the gene with a change of one amino acid in the transmembrane and cytosolic regions of the RhD polypeptide, as opposed to partial D-variants, which are primarily the result of a hybrid gene in which, by gene conversion, a part of the RhD polypeptide is replaced by a part of the RhCE polypeptide in the regions exposed on the outer surface of erythrocytes [17]. There is a considerable number of RHD alleles that encode variants of the D antigen, and some RHD alleles do not have any at all expression of functional antigen D. Although it is most Caucasians RhD positive or negative, there is variation in D expression antigens that we collectively call D variants. They include the D antigens that have complete but weakly expressed D antigens, as well as partial D antigens lacking D epitopes. Most encode single amino acid changes [18].

Others alter the topology and D epitopes of the RhD membrane, while others only alter the quantity of RhD protein in the membrane. These latter are in charge of RhD-positive people who exhibit anti-D antibodies following transfusion or pregnancy. The frequency of several D variations varies considerably amongst groups. With currently available commercial anti-D reagents, it is challenging to distinguish between specific types of weak and weak and partial types, but it is crucial to do so since certain, but not all, types of D variations are responsible for the development of RhD immunization in RhD negative patients [19, 20].
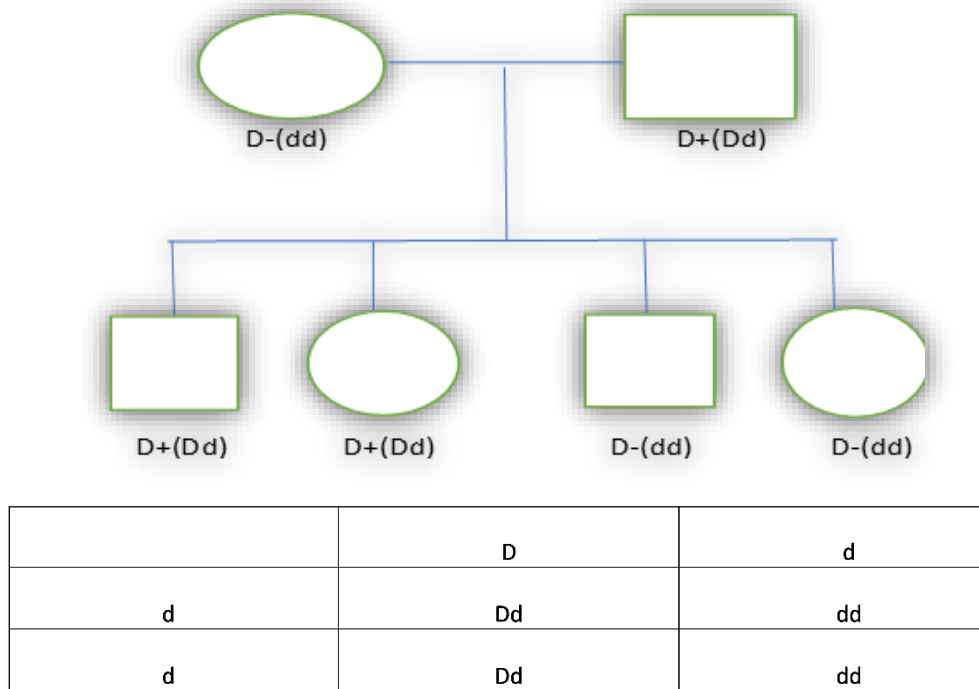
	D	d
d	Dd	dd
d	Dd	dd

Figure 2.Inheritance of the D antigen [21].

The representation of predicting the probability of D-positive offspring from a D-negative mother and a heterozygous (Dd) father. The d gene does not exist and it is used only as an illustration. This mating demonstrates that 50% of children could be D-positive [21].

Various molecular bases of the formation of certain D variants have been described. Weak D antigen is caused by a single mutation of the RHD gene that leads to the replacement of amino acids in the intracellular or transmembrane segment of the RhD protein, resulting in differences in the number of D antigens on the surface of erythrocytes. It comprises 10,000 to 30,000 antigenic sites in the normal Rh phenotype [22].

The DEL phenotype is a unique sort of weak D antigen in which the D antigen is only very weakly expressed serologically [23]. RHD/CE hybrids, single or multiple mutations in the gene sequence encoding the extracellular portion of the D antigen, and these changes in the D antigen's quality serve as the molecular basis for the creation of incomplete D antigens [24]. Serological testing on those who contain these variations may show them as antigenic negative, mildly positive, or positive. DNB is the most prevalent partial type of D antigen in the population of Europe. DVI is clinically significant since it frequently results in HBFN and RhD immunization [25].

D-variants with weak expressions quantitatively in serological testing were formerly thought to have the whole D antigen, with all epitopes [25]. Since the anti-D monoclonal antibody may not react with erythrocytes due to the low avidity of the antibody rather than a lack of binding epitopes, it might be challenging to determine in practice. The main molecular mechanism of RHD alleles encoding the weak D one phenotype is one or more substitutions (wrong mutations), SNVs, with a very small number of duplications and insertions without changing the reading frame. All amino acid substitutions are considered to be present in the membrane and/or cytoplasmic domains of the RhD protein. [26, 27]

But for some weak ones, antigen D the accuracy of that theory depends on whether the depletion in the RhD protein is assumed to model exclusively extracellular or also passes through the membrane [26, 27].

Weak antigens D types 1, 2, and 3 are most common in populations of European origin and together they account for up to 90% of all weak D antigens in a certain population. The frequency of each type of weak antigen D is different between individual populations [28].

Weak D is a variation where the expression of D antigens is significantly diminished but the number of epitopes is normal, as already mentioned. Additionally, there is partial D, a variety where the expression is normal but fewer epitopes are present. Approximately 10,000–30,000 D antigens are present in healthy Rh (D) positive individuals, and weak D erythrocytes contain between 70 and 4,000 D antigens [29]. Direct agglutination can

capture weak D antigens but not partial ones. DIIIa, DVI, DBT, and DFR are examples of partial D phenotypes that can produce anti-D antibodies and be identified serologically [30].

90% of weak D phenotypes, including weak D types 1, 2, 3, and 4, are molecularly specified and, for the most part, do not produce anti-D antibodies [31].

There are exceptions such as the weak D type (4.2, 5, 11- 20) which can create anti-D antibodies when given Rh (D) positive blood group [32].

The weak variant of the D antigen that has the smallest number of antigenic sites is DEL I which is associated with the presence of the mutated RHD gene. In Europe, there are between 1:350 and 1:2,000 donors with DEL [33]. While weak D-26 erythrocytes with 70 antigen sites can agglutinate with anti-D in the antiglobulin test, the maximum number of antigenic sites in DEL is 36, but in most cases, it is less than 22 and cannot be detected by agglutination assays. [34] Molecular techniques are used to identify DEL and other very weak variations because standard serological approaches cannot detect them.

There are represented main differences between D- variants.

1. Partial D: differently shaped D variant

2. Weak D: Although this gene variant produces the same quantity of D antigen as the regular RhD gene, it has a little mutation that prevents it from reaching the cell surface.

3. DEL D: This gene variation produces less D antigen than normal [35].

2. Methods of detection

To determine the rhD antigen, two methods of detecting this antigen can be used. Those are serological, as well as molecular methods of determination.

2.1 Serological determination

In the field of research, attempts were made to work on the synthesis of monoclonal antibodies in blood type serology as early as the 1970s of the previous century [36]. Antibodies can be classified as monoclonal or polyclonal. Monoclonal antibodies are produced from a single clone of plasma cells called B-lymphocytes. They bind to a single epitope of a single antigen and share the same paratopes. Polyclonal antibodies are proteins released by B-lymphocytes of various cell types that bind to various antigen epitopes or even various antigens that were present in the immunizing agent [37].

Polyclonal immune-related antigens are present in the body. The production and isolation of monoclonal antibodies, for which Milstein and Köhler were awarded the Nobel Prize in 1984, were made possible in 1975 by the discovery of hybridoma cell technology [38].

Mouse antibodies (mAt) are created after injecting an antigen into a mouse and extracting its spleen from which plasma cells are obtained that produce antigen-specific antibodies, by combining these cells with immortal mouse myeloma cells, and further growing these hybrid cells from which antibodies are isolated. Mouse monoclonal antibody production was exceedingly successful, particularly for the MN and ABO blood group systems. The development of monoclonal reagents for antigen D, however, has been put off. This is related to the production procedure, which is far more difficult to complete than the production of ABO monoclonal antibodies because antigen D is already so complex. While mouse hybridomas were utilized for the antigens ABO and MN, it was required to create stable cell lines from human B lymphocytes for antigen D [39].

The majority of therapeutic monoclonal antibodies come from the immunoglobulin G group. IgG1, IgG2, IgG3, and IgG4 are the four subclasses of human immunoglobulin G. Every monoclonal antibody is made up of a certain number of variable regions. Given that it is made up of two heavy and two light polypeptide chains, we can distinguish the variable region of the heavy chain from the variable region of the light chain as well as the constant region of the heavy chain and the constant region of the light chain [40].

Polyclonal antibodies were previously utilized in the diagnosis of antigen D before the development of monoclonal antibodies. Pregnant women who had received the vaccine or people who had been inoculated with erythrocytes that were D-positive and matched for all other clinically important antigens were used to acquire serum for the generation of polyclonal reagents with anti-D antibodies [41].

Limited sources, ethical concerns, inconsistent batch quality, and potential infectiousness were drawbacks of this method of obtaining reagents. Additionally, using a high-protein medium led to more frequently occurring false positive results because of serum factors, such as the coating of erythrocytes as a result of prolonged incubation and evaporation. The benefits of monoclonal reagents over them include their greater precision, higher reactivity than polyclonal reagents, independence from the source, more humane and ethical approach, acceptable cost, consistency and stability of series, and availability in large quantities [42].

Monoclonal antibodies against D and their combinations are used almost exclusively today for the serological determination of RhD.

Despite the availability of powerful standardized monoclonal anti-D antibodies and reasonably quick and straightforward agglutination procedures, many RhD phenotypes cannot be isolated based on these tests. The gold standard for identifying blood group antigens and antibodies against them is serological analysis. These were the first techniques used in pre-transfusion testing, and contemporary transfusion therapy made it practicable [43].

Because serological methods of determining the D antigen have their limitations, it is typical in everyday practice to get inconsistent findings. This is due to the lack of accepted procedures for interpreting serologically weakly expressed D antigen test findings, as well as variations in the way common serological tests are carried out and the monoclonal anti-D reagents that are employed, which agglutinate weak D variants differently [44].

Numerous diverse RHD genes may have an impact on the D epitope and molecule's structure in addition to its level of expression [45]. Given their limitations and the expanding accessibility of molecular biology techniques in medicine, immunohematology is becoming more and more crucial for resolving test results that are in question for blood donors and patients (recipients of blood products), as well as for routinely determining the alleles of specific genes [46].

The drawback of monoclonal reagents is that weak variations of the RhD antigen are not consistently detected by them, leading to discrepancies in interpretation and conflicts when determining the RhD status of people. The choice of which monoclonal reagent combination to use in routine work must be made by expert consensus to avoid disagreements. This is because reagents with weaker serological activity protect the recipient from RhD immunizations, while those with stronger reactivity conserve reserves of RhD-negative blood doses [47, 48].

2.2 Methods of determination of Rh (D) antigen

The only way to identify the Rh (D) blood group is by looking for antigens on the membrane erythrocytes [47]. Rh (D) erythrocytes, or erythrocytes with Rh(D) antigen present in the membrane, are agglutinated by the anti-D test serum. Agglutinates are an indicator of a positive response and reveal that a person is Rh(D) positive [49]. It is important to finish the test in the IAT (Indirect Antiglobulin Test) to determine whether the result is a partial D or a D negative erythrocytes when using the direct technique of determination with anti-D test serum that consists of IgG antibodies if the reaction is negative (lack of agglutinates) [50].

To assess the likelihood of the development and prevention of neonatal hemolytic illness, it is essential to determine whether partial D is present in pregnant women and neonates. It is desirable to utilize a completely automated approach to identify Rh (D) and ABO blood groups to lower the possibility of incorrect transcription of the results or misinterpretation of the data. A monoclonal IgM anti-DVI reagent.

DVI (Rhesus D category VI) is allowed for serologically determining antigen D on fully automated systems. The choice of anti-DVI-monoclonal reagent and the definition of the cut-off value are decisions made by each immunohematology laboratory following the manufacturer's instructions and based on its risk assessment [51]. This primarily means that samples are considered Rh (D)-positive if their agglutination strength is >2+ in the micro-agglutination method or >1+ in the test tube method. Samples should be handled according to the protocols if they exhibit 1+ or less agglutination in the test tube method or 2+ or less agglutination in the micro-agglutination method [52, 53]. To prevent unbalanced findings in the identification of D-variants in the absence of a fully automated system, each sample must be tested twice with the same reagent or twice with two separate monoclonal IgM anti-DVI-reagents of similar affinity. This manner of operation is required to lessen the possibility of reagent cross-contamination and procedural mistakes when done manually [52, 53]. No more testing is necessary when the results are both positive and negative. Preferably, samples with the first ambiguous results should be sent for RhD genotyping. These are samples that have weak serological reactions, non-identical results from several anti-D reagents, and results that diverge from earlier results. Monoclonal reagents that must not detect the DVI-variant (anti-DVI-) by direct agglutination method are used to measure antigen D in patients and pregnant women.

To reduce the possibility of a sample with DVI+ as well as a sample that is DAT (direct antiglobulin test) positive and D-negative being mistakenly interpreted as D-positive, it is crucial to emphasize that antigen D is not determined in pregnant women and patients using the indirect antiglobulin test (IAT) [52,53].

The basic diagnostic procedure in determining the Rh (D) blood group is a serological procedure that is performed by determining the D antigen in the direct agglutination method with monoclonal anti-DVI reagents. If there is no agglutination in the tested sample, the blood group is declared Rh (D) negative, and if strong agglutination is present, the blood group is declared Rh (D) positive. RhD genotyping should be used to solve serologically doubtful results [52, 53].

2.3 Molecular determination

Serological and molecular techniques can be used in combination to help clarify doubts. The final point refers to how all disciplines of medicine have been impacted by the recent exponential rise of molecular diagnostics. DNA genotyping is used in transfusion medicine as an adjunct to or a substitute for serological tests to identify blood groups. A DNA test that focuses on these alterations is similar to serological typing since the majority of antigen polymorphisms are generated by a change in one nucleotide polymorphism in the relevant genes. Genotyping of erythrocyte blood groups is sometimes the only option to permit the transfusion of matched erythrocyte concentrates when test serums are not available [54].

Finding antigen-negative blood preparations can be done effectively by genotyping. Numerous medical fields have found a use for blood group antigen genotyping, including prenatal medicine for HDFN (Hemolytic disease of the fetus and newborn) risk assessment and candidate for RhIg, bone marrow donor selection, for hematological patients with anemia who need continuous transfusion treatment, and oncology patients who receive treatment with monoclonal antibodies that interfere with pretransfusion testing [55].

The use of genotyping in laboratory practice greatly improves the security of testing, and the accuracy of determining RhD antigen, and decreases the misuse of RhIg in women with serologically poorly expressed D antigen [56].

Additionally, the use of genotyping would prevent RhD-negative blood products from being transfused to individuals with weak D types 1, 2, and 3. Future whole genome sequencing will make it possible to identify the genes for many erythrocyte antigens that will be included in patient medical documentation and allow for customized transfusion therapy [57, 58].

RhD genotyping is extensively implemented in the majority of Western nations to assess a patient's RhD status before transfusion therapy to clarify ambiguous results of serological D antigen testing. Combining immunohistological, serological, and molecular testing results in the best diagnostic value for identifying D-variants [59].

The most prevalent D-variants, which have a considerable impact on transfusion management and, according to the work of Ogi et al. from 2011, account for more than 85% of D-variants in our population, including weak D-types 1, 2, and 3, can be identified using RhD genotyping. Weak D-type 1, 2, and 3 carriers are treated as D-positive individuals and given D-positive blood products during transfusions [59].

2.3.1 Purification of nucleic acids using the QIA cube device

The QIAamp DSP Virus Spin Kit is a technique that isolates and purifies viral nucleic acids from biological samples using silica gel membrane technology (QIAamp technology). The intended customers of this product are experts in molecular biology, such as technicians and medical professionals [60].

For use in in vitro diagnostic procedures, utilization of the QIAamp DSP Virus Spin Kit is a standard procedure. Lysis, binding, washing, and elution are the four steps of the QIAamp DSP Virus Spin technique, which can be carried out manually using QIAamp MinElute® columns in a conventional microcentrifuge or automatically utilizing the QIAcube or QIAcube Connect MDx device. Instruments from QIAcube and QIAcube Connect MDx automate the separation and purification of nucleic acids [61].

Up to 12 samples from a variety of sources (including whole blood, platelet-leukocyte interlayer, plasma, serum, cell culture, and tissue culture) can be processed in one technique.Standard QIAGEN nucleic acid isolation kits were used for the isolation process following the manufacturer's guidelines. The lysis of the cell membrane and release of the nucleic acid, followed by the adsorption of DNA on the QIAamp silica-gel membrane of the QIAamp spin column and washing off the membrane, constitute the basis of the isolation principle. Depending on the putty being used and the substance from which we isolate, they would choose the insulation procedure from the device's screen [60, 61].

Lysis, incubation, binding, washing, and elution were all processes that were visible on the screen during isolation. Proteins and interfering chemicals that are not nucleic acids but can interfere with the polymerase chain reaction are eliminated during the isolation procedure.

By washing the membrane-bound DNA with two separate washing buffers and eluting it with buffer for washing, the DNA was purified. Following that, a PCR reaction was used to amplify the extracted DNA [60,61]

2.3.2 Real-time PCR- Applied Biosystems

As noted previously, maternal anti-RhD IgG antibodies (anti-D) that cross the placenta and destroy fetal red blood cells cause anemia in the infant (RBCs). This is referred to as (HDN) [63].

The discovery of cell-free fetal DNA in maternal peripheral blood has increased the possibilities for non-invasive prenatal research (serum or plasma). The real-time PCR method is commonly used to determine RhD status. The target DNA molecule is multiplied while also being simultaneously detected or quantified using RT-PCR-applied biosystems [64].

The process is a modified version of the conventional PCR, and its distinguishing characteristic is the "real-time" monitoring of the intensity of fluorescence activity to expose the amplified DNA as the reaction develops. The quantity of PCR product has a direct relationship with the intensity of the fluorescence that is released during multiplication. Compared to normal PCR, which uses the endpoint method to detect the PCR reaction's result, this method is more innovative. Real-time TaqMan technology makes use of a double-labeled fluorescent probe (segment of single-stranded DNA) as well as a silencer and emitting dye "quencher". The proximity of the silencer renders the color reporter inoperative when the sample's fluorescence is intact [65, 66].

The emitting dye is simultaneously being released, being separated from the suppressor, and having its fluorescence activity increased. As a result of Taq DNA polymerase's 5'-3' nuclease activity, the probe for the complementary target sequence hybridizes during multiplication and degrades as a result [66].

By comparing the emission intensity of the emitting dye to the emission of a reference dye present in the reaction mixture, the fluorescence signal is normalized. The fact that the process is repeated for each cycle has no impact on the product's exponential growth. As the amount of the multiplied product increases from cycle to cycle, so does the fluorescence intensity of the emitting dye [67].

The relationship between the fluorescence signal and the number of multiplication cycles is depicted visually by the amplification curve. The initial cycles of multiplication in which there are no significant changes to the fluorescence signal make up the baseline amplification curves. When a signal is detected above the baseline, the multiplied product is indicated. The number of cycles in which the fluorescence intensity exceeds the threshold intensity is indicated by the threshold cycle (Ct) value. The instrument continually measures the samples' fluorescence, and the program analyzes the information [66, 67].

2.3.3 PCR-SSP (polymerase chain reaction with sequence-specific primers)-RBC

RBC-Ready Gene is a product line created by inno-train for the analysis of erythrocyte blood types using the SSP-PCR technique. These diagnostic tools are thus the perfect addition to serological blood group typing. Depending on the investigation issue, the modular kit design provides a wide range of options for a gradual typing system [69].

Agarose gel electrophoresis is used as per the conventional procedure for evaluation. Each tube contains primers for the amplification of human growth hormone as an internal PCR control (HGH). The amplified form of this positive control must be easily discernible if there is no particular product present after PCR [70].

Benefits of the RBC-Ready Gene System include clarification of questionable RHD/RHCE negative controls; easy and quick test performance; safety with poly transfused patients; and colored primer mixes for greater visibility [69, 70].

2.3.4 RhD genotyping

To clarify patients' and donors' serologically weak D typings, Inno-Train systems can be used for RBC-Ready Gene CDE and RBC-Ready Gene D weak alone or together. Since ambiguous samples are explicitly examined for D categories, D partial, and D weak, they may be readily identified. When a sample has a C or E antigen and is serologically D negative, molecular retyping may occasionally reveal a D positivity (DEL, D weak, or D variant) [69,70].

The RBC Ready Gene D AddOn system recognizes extra RHD sequences and additional D negative alleles, such as DELs, RHDpsi, d(C)es, D-CE(2-9)-D, or W16X, that are not brought on by a loss of the entire RHD gene. The identification of these alleles provides crucial extra data for accurate zygosity typing [70].

2.3.5. FluoGene

Fluogene is a type of molecular diagnostics used to demonstrate the presence of the RHD gene in voluntary blood donors who have already been determined to be Rh-D negative using the serological approach. Testing for thrombocyte, erythrocyte, and HLA genes using the FluoGene approach combines all the benefits of PCR-SSP with the speed of fluorescence detection as the test's result [71].

The CDE kit is utilized in the event of individuals with a clearly expressed D antigen, in which one of the types of incomplete D is determined, as well as specific types of weak D. These test kits can be used to specifically analyze the RHD gene. There are more kits for identifying weak D phenotypes in addition to these. Based on a specific TaqMan probe system found in the FluoVista device, the analysis is conducted. This approach has numerous benefits, including the PCR-SSP method without gel electrophoresis, which examines what is needed in around 90 minutes, no hybridization, instantaneous results display after loading, no risk of post-PCR contamination, very little DNA usage, and user-friendly software [72].

3. Discussion

The major purpose of this review paper was to explain the principle of inheritance of the blood groups, the Rh system of blood groups as well as variants of the D antigen, and their significance, as well as potential dangers if there are incompatible donors. The main focus of the work was the analysis of methods in the application of transfusion medicine for RhD antigen detection.

Progress was also seen with the discovery of the ABO, and Rh systems, as well as why however C, c, D, E, and e are the most prevalent and significant ones. The D antigen and its variants have also been described, and it is known that the D antigen is found in RhD-positive people, who make up around 85% of the population overall, but not in RhD-negative people, who cover up about 15% [73].

The RhD system of erythrocyte antigens is the most complex system of blood groups. It is inherited through two adjacent genes RhD and RhCE. The strongest immunogen in the system is the RhD antigen. In the cases when the RhD antigen is less expressed, serological methods cannot determine whether a person should be treated with RhD-negative preparations. Whether it is a weak RhD variant that can break down the anti-D antibody is determined by molecular testing.

It is important to emphasize the importance of taking medication, as well as regular check-ups in pregnant women who are Rh- and who are carrying an Rh+ child because it can often lead to negative consequences and cause hemolytic disease of the newborn, and in severe cases miscarriage and death of the mother.

Also, Weak D and its importance were well described. Weak antigens D types 1, 2, and 3 are most common in populations of European origin and together they account for up to 90% of all weak D antigens in a certain population. The frequency of each type of weak antigen D is different between individual populations [74]. Continuing with the comparison of serological and molecular methods, we discovered the importance and difference between monoclonal and polyclonal antibodies in serological determination, Rh D detection methods, and the procedures and methods of each of them were described in detail, as well as molecular methods.

4. Conclusions

It is important to emphasize the importance of using serological and molecular methods in transfusion medicine, as well as integrating these two methods. It is crucial to underline the practical aspects of both approaches and to note that many efforts are now being made to enhance the application of molecular methodologies. Molecular techniques, on the other hand, are a validation of certain test results.

The main drawbacks of serology are related to reagent availability or variation in reaction intensity, as well as drawbacks related to interference from antibodies or donor RBC. Additionally, because normal serological procedures cannot detect DEL and other very weak variants, molecular techniques are utilized to identify them.

Due to the advantages of molecular genotyping, such as confirmation typing of serological detection, and the fact that molecular methods can use a variety of DNA testing materials, including blood, urine sediment, buffy coat, and amniocytes, it has been determined that they are more acceptable.

Additionally, the identification and analysis of blood that is negative for particular antigens to which a patient may have developed antibodies, the detection and analysis of antibodies, and the identification of donors who are negative for common antigens are all made possible by molecular methods.

Declaration of competing interest

The authors declare that they have no known financial or non-financial competing interests in any material discussed in this paper.

References

[1] Schwarz, H.P. and Dorner, F,"Karl Landsteiner and his major contributions to haematology", *British Journal of Haematology*, vol.*121, no.4,* pp.556-565, 2003.

[2] Thornton, N.M. and Grimsley, S.P, "Clinical significance of antibodies to antigens in the ABO, MNS, P1PK, Rh, Lutheran, Kell, Lewis, Duffy, Kidd, Diego, Yt, and Xg blood group systems" . *Immunohematology*, vol.*35*, no.3, pp.95-101,2019.

[3] Sylvia Pagán Westphal, „Bad blood' causes hundreds of unnecessary deaths", *'Newscientist.com', 2002*

[4] Daniel Murrell, *M.D.,* „ABO Incompatibility Reaction" *By Helen Colledge , „Healthline.com"* , 2017

[5] Dean L. Bethesda, M.D., „Blood transfusions and the immune system", *„National Center for Biotechnology Information (US)" , 2005*

[6] Rajiv K. Pruthi M.B.B.S., „ Is there a universal blood donor type?" *, Mayoclinic.org, 2022*

[7] [American College of Obstetricians and Gynecologists], The Rh factor: How it can affect your pregnancy? acog.org, 2022

[8] Rae Osborn,„Difference between blood groups A, B, AB and O, 2021

[9] Bibiana Palao, „How blood groups are inherited and why it´s important that you know yours", *Veritasint.com*, 2021

[10] Punam Sachdeva, B. G. Patel, and B. K. Patel, „Drug Use in Pregnancy; a Point to Ponder", *Indian Journal of Pharmaceutical Sciences*, 2009

[11] Mor, G., & Cardenas, I. „The immune system in pregnancy: a unique complexity"*American journal of reproductive immunology, vol. 63 no.6*, pp. 425-433, 2010

[12] Gjerset, George F., Sherrill J. Slichter, and John A. Hansen. "HLA, blood transfusion and the immune system." *Clinics in immunology and allergy* 4, no. 3, 503-534, 1984

[13] Flegel, W. A. „The genetics of the Rhesus blood group system" *Blood Transfus, vol. 5 no.2, pp. 50-57*, 2007

[14] Anstee, David J. "The relationship between blood groups and disease." *Blood, The Journal of the American Society of Hematology* 115, no. 23, 4635-4643, 2010

[15] Learn Serology: Immunohematology Learning Modules, *Canadian Blood Services BloodTechNet Award Program* „Rh system"

[16] Flegel, W. A. „The genetics of the Rhesus blood group system" *Blood Transfus, vol. 5 no.2,* pp. 50-57, 2007

[17] Westhoff, C. M. „The structure and function of the Rh antigen complex"*In Seminars in hematology Vol. 44, No. 1*, pp. 42-50). WB Saunders, 2007

[18] Daniels, G. „Variants of RhD–current testing and clinical consequences" *British journal of haematology, vol. 161 no.4* , pp. 461-470, 2013

[19] Chiu, Mark L., Dennis R. Goulet, Alexey Teplyakov, and Gary L. Gilliland. "Antibody structure and function: the basis for engineering therapeutics." *Antibodies* 8, no. 4. 55, 2019.

[20] Wagner, F. F., Frohmajer, A., Ladewig, B., Eicher, N. I., Lonicer, C. B., Müller, T. H., ... & Flegel, W. A. „Weak D alleles express distinct phenotypes" Blood, *The Journal of the American Society of Hematology, vol. 95 no.8,* pp.2699-2708, 2000

[21] Yonsei University Wonju Campus, „ Rh blood group system" , College of Health Sciences

[22] Dhot, P. S., & Machave, Y. V. „STUDY OF WEAK D PHENOTYPE IN HETEROGENEOUS POPULATION*" Medical Journal Armed Forces India, vol. 54 no.4,* pp. 309-310,1998

[23] Sandler, S. G., Chen, L. N., & Flegel, W. A. „Serological weak D phenotypes: a review and guidance for interpreting the RhD blood type using the RHD genotype" *British journal of haematology, vol. 179 no.1*, pp. 10-19, 2017

[24] Bangham, Jenny. "Writing, printing, speaking: Rhesus blood-group genetics and nomenclatures in the mid-twentieth century." *The British Journal for the History of Science* 47, no. 2, 335-361, 2014

[25] Clausen, Frederik Banch, Merete Berthu Damkjær, and Morten Hanefeld Dziegiel. "Noninvasive fetal RhD genotyping." *Transfusion and Apheresis Science* 50, no. 2, 154-162, 2014

[26] Loann Raud, Marlène Le Tertre, Léonie Vigneron, Chandran Ka, Gaëlle Richard, et al.. Missense RHD SNVs induce weakened D antigen expression by altering splicing and/or protein expression. *Transfusion, vol.61 no.8,* pp.2468-2476, 2021

[27] Flegel, W. A., & Srivastava, K. „Frameshift variations in the RHD coding sequence: Molecular mechanisms permitting protein expression"*Transfusio*n*, vol. 60 no.11*, pp.2737, 2020

[28] Westhoff, Connie M. "Blood group genotyping." *Blood, The Journal of the American Society of Hematology* 133, no. 17, 1814-1820, 2019

[29] Gu, J., Sun, A. Y., Wang, X. D., Shao, C. P., Li, Z., Huang, L. H., ... & Sun, G. M. „Analysis of density and epitopes of D antigen on the surface of erythrocytes from DEL phenotypic individuals carrying the RHD1227A allele" *Blood Transfusion, vol.12 no.2* , pp. 244, 2014

[30] Ying, Yanling, Jingjing Zhang, Xiaozhen Hong, Xianguo Xu, Ji He, and Faming Zhu. "The significance of RHD genotyping and characteristic analysis in Chinese RhD variant individuals." *Frontiers in Immunology* 12, 755661, 2021

[31] Bakanay, Sule Mine, Aysenur Ozturk, Talia Ileri, Elif Ince, Suzan Yavasoglu, Nejat Akar, Zumrut Uysal, and Onder Arslan. "Blood group genotyping in multi-transfused patients." *Transfusion and Apheresis Science* 48, no. 2,257-261,2013

[32] Brar, R. K., Shaiji, P. S., & Sehgal, S. „Testing for weak D Antigen: Spectrum and its applied role in rhesus-negative transfusions in Andaman and Nicobar Islands" *Tzu-Chi Medical Journal, vol. 32 no.2* , pp. 167, 2020

[33] Wagner, F. F. „RHD PCR of D-negative blood donors" Transfusion Medicine and Hemotherapy, vol. 40 no.3 , pp. 172-181, 2013

[34] Zhang, Xu, Guiji Li, Zhuren Zhou, Chaopeng Shao, Xuying Huang, Lichun Li, Xiaofeng Li, Ying Liu, Hua Fan, and Jianping Li. "Molecular and computational analysis of 45 samples with a serologic weak D phenotype detected among 132,479 blood donors in northeast China." *Journal of Translational Medicine* 17, no. 1, 1-11, 2019

[35] McGowan, E. C., G. H. Lopez, C. M. Knauth, Y-W. Liew, J. A. Condon, Lanny Ramadi, Karyn Parsons, E. M. Turner, R. L. Flower, and C. A. Hyland. "Diverse and novel RHD variants in Australian blood donors with a weak D phenotype: implication for transfusion management." *Vox Sanguinis* 112, no. 3, 279-287, 2017

[36] Liu, J. K. „The history of monoclonal antibody development–progress, remaining challenges and future innovations" *Annals of medicine and surgery, vol. 3 no. 4* , pp.113-116, 2014

[37] Mitra, S., & Tomar, P. C. „Hybridoma technology; advancements, clinical significance, and future aspects" *Journal of Genetic Engineering and Biotechnology, vol. 19 no. 1,* pp. 11-12, 2021

[38] Freysd'ottir, J. O. „Production of monoclonal antibodies"*Diagnostic and Therapeutic Antibodies*, pp. 267-279, 2000

[39] Parray, Hilal Ahmed, Shivangi Shukla, Sweety Samal, Tripti Shrivastava, Shubbir Ahmed, Chandresh Sharma, and Rajesh Kumar. "Hybridoma technology a versatile method for isolation of monoclonal antibodies, its applicability across species, limitations, advancement and future perspectives." *International immunopharmacology* 85, 106639, 2020

[40] Vidarsson, G., Dekkers, G., & Rispens, T. „ IgG subclasses and allotypes: from structure to effector functions"*Front Immunol. 5: 520*, 2014

[41] Buss, Nicholas APS, Simon J. Henderson, Mary McFarlane, Jacintha M. Shenton, and Lolke De Haan. "Monoclonal antibody therapeutics: history and future." *Current opinion in pharmacology* 12, no. 5, 615-622, 2012

[42] Nelson, P. N., Reynolds, G. M., Waldron, E. E., Ward, E., Giannopoulos, K., & Murray, P. G." Monoclonal antibodies"*Molecular pathology: MP, vol. 53 no.3,* pp. 111-117, 2000

[43] Li, H. Y., & Guo, K. „Blood Group Testing"*Frontiers in Medicine*, 9, 2022

[44] Lukačević Krstić, J., Dajak, S., Bingulac-Popović, J., Dogic, V., & Mratinovic-Mikulandra, J. „Anti-D reagents should be chosen accordingly to the prevalence of D variants in the obstetric population" *Journal of Clinical Laboratory Analysis, vol. 32 no.3*, e22285, 2018

[45] Reid, Marion E., Christine Lomas-Francis, and Martin L. Olsson. *The blood group antigen factsbook*. Academic press, 2012.

[46] Morelati, F., Barcellini, W., Manera, M. C., Paccapelo, C., Revelli, N., Villa, M. A., & Marconi, M. „New technologies in immunohaematology" *Blood Transfusion, vol. 5 no.2,*pp. 58, 2007

[47] Figueroa, Dolores. "The Diego blood group system: a review." *Immunohematology* 29, no. 2 , 73-81, 2013

[48] Kumar, H., Mishra, D. K., Sarkar, R. S., & Jaiprakash, M. „Difficulties in immunohaematology: The weak D antigen"*Medical Journal Armed Forces India, vol.61 no.4* pp.348-350, 2005

[49] Malomgré, Wim, and Birgid Neumeister. "Recent and future trends in blood group typing." *Analytical and bioanalytical chemistry* 393, no. 5, 1443-1451, 2009

[50] Wagner, Franz F., Christoph Gassner, Thomas H. Müller, Diether Schönitzer, Friedrich Schunter, and Willy A. Flegel. "Molecular Basis of Weak D Phenotypes: Presented at the 25th Congress of the International Society of Blood Transfusion held in Oslo on June 29, 1998 and published in abstract form in Vox Sang 74: 55, 1998 (suppl)." *Blood, The Journal of the American Society of Hematology* 93, no. 1, 385-393,1999

[51] Wagner, F. F., Gassner, C., Müller, T. H., Schönitzer, D., Schunter, F., & Flegel, W. A. „Three molecular structures cause Rhesus D category VI phenotypes with distinct immunohematologic features" Blood, *The Journal of the American Society of Hematology, vol. 91 no.6* , pp. 2157-2168, 1998

[52] Safić Stanić Hana," RHD genotyping relevance in RhD negative blood donors", Ph.D. Dissertation Thesis; „Vaznost genotipizacije u RhD negativnih darivatelja krvi, Sveuciliste u Zagrebu , Medicinski fakultet Zagreb, Doktorska disertacija, 2021

[53] Marko Lilic „ Molecular identification of RhD alleles in serollogicaly RhD negative blood donors from Northwestern Croatia" Ph.D. Dissertation Thesis; „ Molekularno odredjivanje alela RhD u populaciji seroloski RhD negativnih davatelja krvi Sjeverozapadne Hrvatske, Sveuciliste Josipa Jurja Strossmayera u Osijeku, Doktorska disertacija Sveuciliste u Dubrovniku Institut Rudjer Boskovic, 2022

[54] Dwivedi, S., Purohit, P., Misra, R., Pareek, P., Goel, A., Khattri, S., ... & Sharma, P. „ Diseases and molecular diagnostics: a step closer to precision medicine" *Indian Journal of Clinical Biochemistry, vol. 32 no.4*, pp. 374-398, 2017

[55] Hussein, Eiman, and Jun Teruya. "Weak D types in the Egyptian population." *American journal of clinical pathology* 139, no. 6 (2013): 806-811.

[56] Perez-Alvarez, Ingrid, Chelsea Hayes, Tiruneh Hailemariam, Edward Shin, Tyler Hutchinson, and Ellen Klapper. "RHD genotyping of serologic RhD-negative blood donors in a hospital-based blood donor center." *Transfusion* 59, no. 7, 2422-2428, 2019

[57] Bakanay, Sule Mine, Aysenur Ozturk, Talia Ileri, Elif Ince, Suzan Yavasoglu, Nejat Akar, Zumrut Uysal, and Onder Arslan. "Blood group genotyping in multi-transfused patients." *Transfusion and Apheresis Science* 48, no. 2 (2013): 257-261.

[58] Sandler, S. G., Flegel, W. A., Westhoff, C. M., Denomme, G. A., Delaney, M., Keller, M. A., ... & Simon, C. D. „It's time to phase-in RHD genotyping for patients with a serological weak D phenotype" *Transfusion, vol. 55 no.3*, pp. 680, 2015

[59] Flegel, W. A. „Proceed with care: the "uncommon" serologic weak D phenotypes*"Blood Transfusion, vol. 19 no. 4* , pp.272, 2021

[60] QIAamp® DSP Virus Spin Kit Instructions for Use (Handbook), 2021

[61] QIAamp® DSP Viral RNA Mini Kit Instructions for Use (Handbook), 2021

[62] Nassar, G. N., & Wehbe, C. „ Erythroblastosis fetalis", 2018

[63] Bohmova, J., Lubusky, M., Holuskova, I., Studnickova, M., Kratochvilova, R., Krejcirikova, E., ... & Vodicka, R. „Two reliable methodical approaches for non-invasive RHD genotyping of a fetus from maternal plasma" *Diagnostics, vol. 10 no. 8*, pp. 564, 2020

[64] Liu, H., Wang, H., Shi, Z., Wang, H., Yang, C., Silke, S. & Lu, Z. „TaqMan probe array for quantitative detection of DNA targets." *Nucleic acids research, vol. 34 no. 1*, e4-e4, 2006

[65] Applied Biosystems Real-Time PCR Systems, Reagent Guide, 850 Lincoln Centre Drive, 2010

[66] Sciellour, Christelle Rouillac-Le, Philippe Puillandre, Rolande Gillot, Céline Baulard, Sylvain Métral, Caroline Le Van Kim, Jean-Pierre Cartron, Yves Colin, and Yves Brossard. "Large-scale pre-diagnosis study of fetal RHD genotyping by PCR on plasma DNA from RhD-negative pregnant women." *Molecular Diagnosis* 8, 23-31, 2004

[67] RBC-Ready Gene THE SSP-PCR SYSTEM certified blood group genotyping, Niederhöchstädter Straße 62 D-61476 Kronberg/Taunus Germany (handbook)

[68] RBC-Ready Gene, inno-train.de, handbook.

[69] Hart, S., C. M. Cserti-Gazdewich, and S. A. McCluskey. "Red cell transfusion and the immune system." *Anaesthesia* 70, 38-e16, 2015

[70] Nathalang, Oytip, Kamphon Intharanut, Wiradee Sasikarn, Siriporn Nathalang, and Pawinee Kupatawintu. "A new polymerase chain reaction: sequence-specific primer method for the Augustine blood type." *Blood Transfusion*, vol 14 no. 6, 577, 2016

[71] Guzijan, Gordana, Snezana Jovanovic Srzentic, Natasa Pavlovic Jankovic, Iva Djilas, and Marko Lilić. "Implementation of molecular RHD typing at two blood transfusion institutes from southeastern Europe." *Transfusion Medicine and Hemotherapy* 46, no. 2, 114-120, 2019

[72] RBC FluoGene - Blood group typing Catalog Hematology and blood bank Blood Group Genotyping (RBC), dlongwood.com

[73] Keller, Jessica A., Trina Horn, Sabine Scholz, Sabrina Koenig, and Margaret A. Keller. "Comparison of genotyping methods: a study of two low-resolution polymerase chain reaction assays in a clinical testing laboratory." *Immunohematology* 35, no. 4: 149-153.

[74] Kacker, Seema, Ralph Vassallo, Margaret A. Keller, Connie M. Westhoff, Kevin D. Frick, S. Gerald Sandler, and Aaron AR Tobian. "Financial implications of RHD genotyping of pregnant women with a serologic weak D phenotype." *Transfusion* 55, no. 9, 2095-2103, 2015