Molecular versus serological techniques in characterization of Rh D variants: Role in improving Rh D typing strategy

Thesis

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Abstract

The Rh blood group system is the most polymorphic of the human blood groups, consisting of at least 50 independent antigens and, next to ABO, is the most clinically significant in transfusion medicine **(Avent & Reid, 2000).**

The D antigen is the most immunogenic antigen of the Rh system. About 18% of Caucasians do not express an antigen D (Rh negative) and most Rh-negative cases are caused by RHD gene deletion. About 0.2-1 % of Caucasians have red blood cells with reduced expression of the D antigen **(Lin et al., 2003)**.

The antibodies in the Rh blood group system can cause severe transfusion reactions and are second only to the ABO system in this regard. Blood grouping by serology has been an enormously successful technology which has made transfusion safe. But the experts in the field are well aware that blood incompatibility remains a significant problem in transfusion medicine and that these problems reflect certain inherent limitations of hemagglutination based testing ***(Antonella et al., 2009).***

The molecular genetics world was revolutionized in 1983 with the advent of PCR (polymerase chain reaction), which allows the amplification of DNA and analysis of genes. The molecular identification and characterization of blood group genes revealed that the majority of clinically important blood group antigens are encoded by SNPs **(Daniels, 2009)**.

The aim of this study is to investigate the role of RHD molecular typing technique in solving RHD typing discrepancies during routine testing due to partial D or weak D phenotypes. Compare currently used serologic methods with molecular analysis to determine the potential application of molecular methods to improve RHD typing strategies.

Key words: Rh- serological techniques- molecular techniques- RhD variants

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LIST OF ABBREVIATIONS

AHG	*Anti human globulin*
AIHA	*Auto immune hemolytic anemia.*
ASP-PCR	*Allele-specific polymerase chain reaction*
cDNA	*Complementary DNA*
CML	*Chronic myeloid leukemia*
DAT	*Direct Antiglobulin testing*
epD	*Epitope D*
FMH	*Feto-maternal hemorrhage*
FRET	*Fluorescence resonance energy transfer*
FUT	*Fucosyl transferase*
gDNA	*Genomic DNA*
GPI	*Glycosylphosphatidylinositol*
HDFN	*Hemolytic disease of fetus and newborn*
HGH	*Human growth hormone*
HTR	*Hemolytic transfusion reaction*
IAT	*Indirect Antiglobulin testing*
ISBT	*International Society of Blood Transfusion*
MPX	*Multiplex*
PCH	*Paroxysmal Cold haemoglobinuria.*
RFLP	*Restriction fragment length polymorphism*
Rh	*Rhesus*
RhAG	*Rhesus Associated glycoprotein*
SMP1	*Small Membrane protein*
SNP	*Single nucleotide polymorphism*
SSP	*Sequence specific primer*
TBE	*Tris-borate EDTA buffer*
UV	*Ultra violet*

Introduction and Aim of the work

Blood grouping by serology has been an enormously successful technology which has made transfusion safe. But the experts in the field are well aware that blood incompatibility remains a significant problem in transfusion medicine and that these problems reflect certain inherent limitations of hemagglutination based testing. These include the weak reactivity of certain clinically significant antibodies, weak expression of some red cell antigens, the lack of universal test methods for antibody detection and identification and the subjective nature of the tests performed. Additionally, there are a number of issues related to reagents themselves, and these also are expressed as technology limitations and they include, of course, the variability in the reagents, the lack of reagent grade antibodies and the different reactivity of monoclonal antibodies compared to polyclonal antibodies ***(Antonella et al., 2009).***

The Rh blood system is one of the most polymorphic and immunogenic systems known to humans. The expression of Rh blood group antigen is complex. The Rh D antigen is the most important of the antigens that constitute the Rh antigen system. In most cases, D antigen can easily be detected. However, due to variability of expression, weak form antigens are encountered. The reactivity of weak D with antisera is variable and presents as a problem in blood banking ***(Mariza et al., 2009).***

In a study by ***Hillyer et al 2008*** that addressed the integration of molecular technologies for red blood cell typing and compatibility testing into blood centers and transfusion services was suggested, however they proposed further clinical assessment of genotyping versus phenotyping, potential role of genotyping to replace pretransfusion testing as well as some practical considerations like cost effectiveness, limitations, who to genotype and quality assurance. Such suggestions open the way for further investigation of the molecular role in different red cell antigen typing.

The Rh antigens are coded for by two genes, RHD and RHCE. These two genes, each of about 60 kilobases, are located in positions 1p34 - 1p36 of the short arm of chromosome 1 and formed of 10 exons. The RHD genes codes for the D antigen, while the RHCE gene codes for the C or c and E or e antigens. More than 50 other antigens have been described in addition to the D,C,c,E,e antigens; these antigens have been identified from their corresponding antibodies and are classified as low or high frequency antigens ***(Peng et al.,2003).***

The D antigens and those of the CE series are transmembrane proteins of 417 amino acids, which act as ammonium transporters. They differ by a small number of amino acids in loops 3, 4, and 6 of the protein. The most important is the D antigen, a mosaic of numerous antigenic determinants or "epitopes". Originally, 9 epitopes were identified, but subsequent serological investigations have distinguished at least 37 different models of reaction with monoclonal anti-D antibodies ***(Judd et al., 2005).***

The vast majority of people from all ethnic backgrounds demonstrate very strong hemagglutination with modern anti-D reagents regardless of the testing methodology. However, this is not always the case. "Weak D" RBCs demonstrate reduced quantities of the D antigen because of mutations in the protein's transmembrane domains. As the name implies, these RBCs tend to demonstrate either weak or no hemagglutination with anti D reagents, although they sometimes react more strongly in the weak D test. "Partial D" RBCs, a phenomenon less common than weak D, usually contain normal numbers of RhD protein, although the protein is mutated, eliminating at least one D-specific epitope on the RhD protein. Weak D and partial D blood recipients/pregnant women might become sensitized to the D antigen if exposed to D positive RBCs ***(Theochari et al., 2009).***

Several assays for blood group genotyping of patients and donors have recently been developed to predict the blood group antigen profile of an individual, with the goal of reducing risk or helping in the assessment of the risk of hemolytic disease of the newborn (HDN) and hemolytic transfusion reactions. They include PCR-RFLP, allele-specific PCR, sequence-specific PCR (SSP-PCR) as single or multiplex assays, real-time quantitative PCR ***(Wagner et al., 2000).***

These assays can be applied to blood group antigens to type patients who have recently received transfusions; to type patients whose RBCs are coated with immunoglobulin; to identify a fetus at risk for HDN; to determine which phenotypically antigen negative patients can receive antigen-positive RBCs; to type donors for antibody identification panels; to type patients who have an antigen that is expressed weakly on RBCs; to determine *RHD* zygosity; to mass screen for antigen-negative donors; to resolve A, B, and D discrepancies ***(Wagner et al., 2000).***

Aim of the work:

The aim of this study is to investigate the role of RHD molecular typing technique in solving RHD typing discrepancies during routine testing due to partial D or weak D phenotypes. Compare currently used serologic methods with molecular analysis to determine the potential application of molecular methods to improve RHD typing strategies.

Red Blood Cell Antigens

Blood group antigens are surface markers on the red blood cell membrane and could be defined as; an inherited character of the red cell surface, detected by a specific alloantibody. Before the 1900s, it was thought that all blood was the same, a misunderstanding that led to frequently fatal transfusions of animal blood into humans and hazardous transfusions of blood between people. Blood groups do not have to be red cell specific, or even blood cell specific, and most are also detected on other cell types **(Daniels and Bromilow, 2011).**

❖ Structure of Red blood cell antigens:

The structures of the different blood group systems and their antigens have been studied extensively particularly since the development of molecular genetic techniques. Blood group antigens may be:

- Proteins,
- Glycoproteins, with the Antibody recognizing primarily the polypeptide backbone;
- Glycoproteins, with the Antibody recognizing the carbohydrate moiety;
- Glycolipids, with the Antibody recognizing the carbohydrate portion.

Blood group proteins and glycoprotiens are integral structures of the red cell membrane. Diagrammatic representations of some blood group proteins and glycoprotiens in the membrane are shown in **Figure 1 (Daniels and Bromilow, 2011).**

Red Blood Cell Antigens

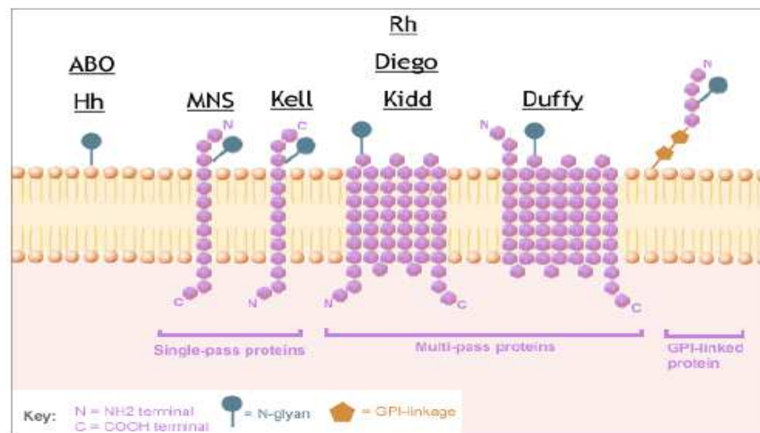

Fig.1 shows **the red blood cell membrane** and some of the blood group antigens attached to it.

Some pass through the membrane once. These generally have an external N-terminal domain and a cytoplasmic C- terminal domain **(type 1)**, though in one case (the Kell glycoprotein) the C-terminus is external and the N-terminus is internal **(type 2).** Some are polytopic **(Type 3)**; that is they cross the membrane several times. Usually both termini are cytoplasmic but the Duffy glycoprotein has an odd number of membrane-spanning domains and an extracellular N-terminal domain. Finally, some have no membrane-spanning domain, but are anchored to the membrane by a lipid tail (called a glycosylphosphatidylinositol or GPI anchor), which is attached to the C-terminus of the protein through carbohydrate **(Type 5)**. There are no Type 4 glycoproteins, which have no external domain, in the red cell membrane. Most red cell surface proteins are glycosylated, the only exceptions being the Rh and Kx proteins. This glycosylation may be **(1)** N-glycosylation, large, branched sugars attached to asparagine residues of the amino acid backbone or **(2)** O-glycosylation, smaller glycans (usually tetrasaccharides) attached to serine or theronine residues **(Daniels and Bromilow, 2011)**.

❖ Blood group antibodies:

Blood groups are antigens and, by definition, a molecule cannot be antigen unless it is recognized by an antibody (or T-cell receptor). So all blood group specificities are defined by antibodies. Most adults have antibodies to the A or B antigens, or to both; that is, they have naturally occurring anti-bodies to those ABO antigens they lack. For most other blood groups corresponding antibodies are not naturally occurring, but are only formed as a result of immunization by transfused red cell or by fetal red cells leaking into the maternal circulation during pregnancy or childbirth. Blood group antibodies are usually IgM or IgG, although some may be IgA. Naturally occurring antibodies are usually predominantly IgM, whereas immune antibodies are predominantly IgG. As a general rule, IgM antibodies will directly agglutinate-positive red cells in a saline medium, whereas most IgG antibodies require potentiators or anti-human globulin to effect agglutination **(Daniels and Bromilow, 2011)**.

❖ Clinical importance of blood groups:

Blood groups are of great clinical importance in blood transfusion and in transplantation. In fact, the discovery of the ABO system was one of the most important factors in making the practice of blood transfusion possible. Many blood group antibodies have the potential to cause rapid destruction of transfused red cells bearing the corresponding antigen giving rise to a hemolytic transfusion reaction (HTR) either immediately or several days after the transfusion **(Daniels and Bromilow, 2011)**.

At their worst HTRs give rise to disseminated intravascular coagulation, renal failure and death. At their mildest they reduce the efficacy of the transfusion. IgG blood group antibodies can cross the placenta during pregnancy and hemolyse fetal red cells expressing the corresponding antigen. This may cause alloimmune fetal hemolytic anemia, more commonly know as hemolytic disease of the fetus and newborn (HDFN). Many blood group