Molecular biology methods for blood cell antigen genotyping in reference laboratories

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Summary
The aim of the study is to present current applications of molecular biology methods in immunohematology as well as the status of their implementation in the Polish Blood Transfusion Service. Molecular biology methods are used for: analysis of molecular background of blood cell antigens in individuals with discrepant results of serological tests, identification of RhD antigen with weak expression undetectable by serological methods in blood donors, identification of weak D type antigen in recipients unsusceptible to alloimmunization. These methods are also used for noninvasive study of fetal genes in plasma samples of immunized pregnant women with serological incompatibilities and in RhD negative pregnant women for qualification to antenatal prophylactic administration of anti-D immunoglobulin. Molecular biology is also an important tool for diagnostics of patients with antibodies to high-frequency blood group antigens (HFA) or for patients immunized with HPA and HNA antigens and for identification of “antigen-negative” blood donors for such patients. In Poland, all these tests are available and in use.

Key words: molecular methods for genotyping, blood group antigens, platelet antigens (HPA), granulocyte antigens (HNA), immunohematological studies

Introduction
Molecular typing of blood cell antigens has been steadily developing since the first reports on DNA sequences of genes coding the clinically significant blood cell antigens which were published in 1989/1990. Since mid-1990s the methods were gradually introduced into the diagnostic process [1–4], initially for platelet and granulocyte antigen typing, where serological methods proved inadequate [4–8].

Since the beginning of 2000, molecular biology methods have also been applied to red blood cell antigen typing, which was made possible due to successful studies of genetic background of blood group systems [9–11].

Current status of application of molecular biology methods in reference laboratories in Poland and worldwide

At present, both in Poland and worldwide, DNA-based methods are used mainly in highly specialized immunohematology reference laboratories [9–14].

Methods are based on the polymerase chain reaction (PCR). The earliest developed PCR-based assays rely on the amplification using sequence specific primers (PCR-SSP) and pattern analysis of the PCR products in agarose gel or on the electrophoretic analysis of restriction fragment length polymorphism (PCR-RFLP).
after digestion of PCR products with restriction enzymes matched to the alleles. A more recent modification of this technique is PCR based on fluorescent staining of the amplification product, i.e., real-time PCR with allele specific TaqMan or HybProbe probes or with SYBR Green DNA intercalating dyes. Real-time PCR methods based on the analysis of the amplification product melting curve (HRM) are also used (Table 1) [12–14]. The blood transfusion service in the Netherlands makes use of a technique based on Multiplex Ligation-dependent Probe Amplification (MLPA) [15]. A growing number of centers is beginning to make use of technologies designed for larger-scale research, including technologies based on microarrays, mass spectrometry, digital PCR. There is also ongoing research on next generation sequencing (NGS). The above issues have been described by the authors in separate papers published in 2019 [16, 17].

Genotyping studies are based on the so called home made tests or, if available, on standardized IVD marked (in vitro diagnostics) commercial tests. Table 2 presents a list of such tests which also appears in Guz et al. 2019 [16].

If above described methods give no equivocal answers with regard to molecular basis of the antigen, additional tests are necessary which are mainly based on sequencing the antigen-encoding gene. The classical Sanger method is usually used, but such analyses can also be performed with next-generation sequencing (NGS) [17].

The IVD commercial tests listed in Table 2 and used for consultation testing at the Institute of Hematology and Transfusion Medicine (IHTM) are “low throughput” tests for single-sample testing. On the other hand, developed at the IHTM real-time PCR home made tests with automated DNA isolation and automated preparation of the mixture of reagents for PCR have “average throughput” which depends on the size of the thermal block in real-time PCR thermocycler and usually analyzes 96–384 samples for one polymorphism at a time. Such tests require an automated DNA isolation device and a pipetting station.

Molecular biology tests currently performed at the IHTM reference laboratory are like those performed in immunohematology laboratories worldwide and focus on:

1. investigation of discrepancy between results of routine serological typing in immunohematology laboratories for determination of ABO and/or Rh group or for matching blood for transfusion;

<table>
<thead>
<tr>
<th>Table 1. Low and medium throughput methods used for blood group genotyping in reference laboratories worldwide:</th>
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<tbody>
<tr>
<td><strong>PCR with agarose gel electrophoretic analysis</strong></td>
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<tr>
<td>PCR-RFLP</td>
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<td>PCR-SSP:</td>
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<tr>
<td>• home made mono/multiplex</td>
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<tr>
<td>• commercial (Inno-Train, BAG Diagnostics)</td>
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<td><strong>Real-time PCR</strong></td>
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<tr>
<td>• TaqMan (home made; FluoGene Inno-Train)</td>
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<td>• Hybprobes</td>
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<td>• HRM</td>
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<td><strong>Capillary electrophoresis</strong></td>
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<td>MLPA</td>
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Abbreviations are explained in the text

2. identification of donors with weak RhD antigen expression, undetectable by serological methods;
3. identification of serologically weak D type 1, 2 or 3 antigens in recipients and pregnant women unsusceptible to anti-D alloimmunization;
4. non-invasive fetal blood antigen genotyping of women with feto-maternal incompatibility;
5. non-invasive fetal RhD typing in RhD-negative women to qualify RhD positive fetus for antenatal anti-D immunoprophylaxis;
6. blood group antigen genotyping in search for “antigen negative” donors for patients with alloantibodies to the high-frequency antigens;
7. HPA and HNA antigen genotyping in diagnostics of cases suspected of alloimmunization and for establishing a registry of donors with typed HPA/HNA antigens for immunized patients.

We present below the application areas for molecular biology methods.

1. **Investigation of discrepancies between results of routine serological typing, mainly difficulties in ABO and Rh typing, and matching blood for transfusion**

A detailed list of application areas for molecular biology methods is presented in points 1, 2 and 3 of Table 3. Molecular biology tests are always preceded by serological tests performed at the IHTM reference laboratory. Based on the results of serological tests, the Laboratory of Blood Group Genetics decides on the choice of molecular biology technique using different tests detailed in Table 2. [18–20].

2. **Identification of donors with weak RhD antigen expression undetectable by serological methods**
It is known from the literature that blood of serologically negative donors with weak D antigen expression may be immunogenic and induce the formation of immunogenic alloantibodies in the recipient [21, 22]. The IHTM has developed a system of identifying such donors [23, 24] based on RHD gene detection in DNA isolated from a plasma pool of serologically RhD negative donors. On RHD gene detection further tests are performed in cross pools or individual samples to identify a donor with RHD. Subsequent molecular and serological typing determines whether RHD gene expression occurs i.e. whether the D antigen can be detected by methods more sensitive than the routinely used, adsorption/elution in particular [23, 24]. Detection in pooled plasma samples from many donors markedly reduces the cost of DNA isolation and identification of donors with immunogenic D antigen. The estimated cost is about PLN 50 per donor assuming that each pool consists of samples from 48 RhD negative donors. Such tests are currently performed in several European countries (Germany, Austria, Switzerland), though the strategy in those countries consists in pooling DNA isolated from whole blood [25, 26]. According to literature, 30 antigen D molecules are sufficient to elicit the immune response in a recipient. It therefore follows that formation of anti-D antibodies in a patient may be induced by transfusion of red blood cell concentrate from a donor with weak antigen D expression. Alloimmunization may be induced even by transfusion from a donor with DEL type antigen which is detected only by adsorption/elution not routinely performed [22].

The IHTM program financed by the National Science Center (NCN) demonstrated that the potentially immunogenic D antigen is detected in approximately 0.09% of donors routinely typed as D negative. All identified donors were with C+ and/or E+ phenotypes [23]. For greater safety of RhD negative recipients, RhD negative C+ and/or E+ donors should be genetically tested, and donors with RHD gene variant encoding D antigen expression should be qualified as RhD positive donors. Appropriate research methodology has been developed and the program is ready for implementation. The estimated cost of testing a plasma pool of 24 negative Rh donors with the C+ or E+ phenotype is estimated at approximately PLN 80 per donor.

3. Identification of weak D type 1, 2 or 3 antigen in recipients and pregnant women unsusceptible to alloimmunization

Implementation of molecular biology methods for analysis of the RHD gene in patients with RhD antigen undetermined in serological tests, led to identification of D variant antigens [12, 13, 27]. Long-term observations have demonstrated that individuals with weak D type 1, 2 or 3 antigens do not produce anti-D alloantibodies despite exposure to RhD-positive blood (antigen incompatible fetus or transfused blood). If anti-D antibodies are detected, they are always autoantibodies [27]. Therefore, individuals with such weak D antigen variants may be transfused with red blood cells from positive RhD donors, and women do not require anti-D immunoglobulin after miscarriage or delivery of a RhD positive newborn. Antigen D
alloimmunization has been described in individuals with other weak D antigen variants, such as weak D type 4.2 (DAR), 11 and 15 [27, 28].

The IHTM methodology for genotyping antigen D variants in individuals with discrepant or weak positive results of serological typing enabled the assessment of the frequency of weak or partial D antigen variants in Poland. The 2019 data of 693 individuals with inconclusive results demonstrated that alleles responsible for weak antigen D type 1, 2, 3 were detected in 79% and in the remaining 21% alleles susceptible to immunization with antigen D were identified (defining weak D, partial D or DEL antigen) [29].

Previous estimates of blood donors only have revealed that routine serological antigen D typing is unequivocal in 0.2% (20/10 000) and most of them (14/10 000) were also genetically defined as RH*D*01W1, RH*D*01W2, RH*D*01W3, i.e. unsusceptible to alloimmunization [30].

4. Non-invasive fetal genotyping of women with feto-maternal incompatibility

The IHTM developed and implemented non-invasive fetal typing of RHD and RHCE genes in women with feto-maternal incompatibility for D, C, c and E antigens. IHTM performs prenatal diagnostics for immunized women with anti-D (+C), anti-c, anti-E or anti-G antibodies from all over the country. Standardization studies demonstrated a 100% compliance of the newborn phenotype/genotype with fetal DNA genotype from maternal plasma. Ongoing are standardization studies on KEL*1 allele in plasma of pregnant women with anti-K alloantibodies [31–36].

5. Non-invasive fetal RHD genotyping of RhD negative pregnant women for qualification to antenatal anti-D prophylaxis

Several Western European countries have implemented routine non-invasive fetal RHD genotyping in maternal plasma of RhD negative pregnant women as criteria for antenatal anti-D prophylaxis for those with RHD positive fetus [37–40]. The program generated 40% savings of anti-D immunoglobulin which counterbalanced the cost of program implementation. It is worth emphasizing that due to the screening program women carrying a RHD negative fetus are not exposed to unnecessary administration of anti-RhD immunoglobulin, which is produced from plasma of blood donors immunized with RhD positive red blood cells. The IHTM has developed a system for large-scale screening [41, 42]. Automation of the process of DNA isolation from plasma and preparation of PCR reactions for fetal RHD

Table 3. Molecular biology methods in immunohematology of blood groups

<table>
<thead>
<tr>
<th>1. Determination of phenotype:</th>
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<tr>
<td>• in patients after multiple transfusions: two red blood cell populations up to 3 months following transfusion</td>
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<tr>
<td>• when blood cells are coated with IgG autoantibodies (positive direct anti-globulin test — DAT)</td>
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<tr>
<td>• when suspected immunization with common or rare antigens</td>
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<td>• when no diagnostic sera are available (e.g. anti-VS, -Yb; -Js, -Vel, -Co)</td>
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<tr>
<td>2. Assistance in the identification of immunogenic antibodies:</td>
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<td>• determination of the presence/absence of the antigen corresponding to suspected specificity of antibodies in case of ambiguous serological results</td>
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<tr>
<td>• help in distinguishing allo- from auto-antibodies by determining the patient’s genotype/phenotype and indicating phenotype of the reference red blood cells needed for alloadosorption</td>
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<tr>
<td>3. Investigating the causes of discrepancies in serological typing — identifying variants:</td>
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<tr>
<td>• RHD gene with atypical expression of D: weak D, partial D, DEL in Rh negative individuals</td>
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<tr>
<td>• RHCE gene with atypical expression of C/c and/or E/e antigens</td>
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<tr>
<td>• ABO gene</td>
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<tr>
<td>• ACKR1/DARC gene: FYnull (-67C), weak FYb (FY*X)</td>
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<tr>
<td>4. Non-invasive fetal blood group/HPA genotyping from maternal blood:</td>
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<tr>
<td>• in immunized pregnant women with alloantibodies (anti-D, -C, -G, -C, -E, -K, -HPA-1a)</td>
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<tr>
<td>• in non-immunized RhD negative women for qualification to antenatal anti-D prophylaxis</td>
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<tr>
<td>5. Formation of blood donor registries of blood cells panels for identification of antibodies:</td>
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<tr>
<td>• search for homozygotes in C/c, E/e, K, Fya/b, Jka/b, MNS antigens (various combinations)</td>
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<tr>
<td>• search for negative phenotypes in HFAs, i.e: k-, Kp(b-), Js(b-), Lu(b-), Di(b-), Yt(a+), Co(a-), Jr(a-), Lan(-), Vel(-)</td>
</tr>
<tr>
<td>• search for positive phenotypes in LFAs i.e: VS+, Js(a+), Kp(a+), Di(a+), Wr(a+)</td>
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<tr>
<td>• HPA and HNA genotyping: especially fetuses/newborns with alloimmune thrombocytopenia (FNAIT)</td>
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6. Genotyping of blood group antigens in search for ‘high-frequency antigen negative’ donors for immunized patients

Alloimmunization against antigens which are present with the high frequency in the general population (HFA “high-frequency antigens”) is a challenge for transfusion medicine since it is difficult to identify the right donor (with no antigen) for a patient who requires transfusion [43, 44]. Finding the HFA negative blood donor often involves screening of several hundred or even thousand blood donors with serological methods which often prove inadequate due to the lack of diagnostic sera. The staff of the Laboratory of Red Blood Cell Immunology at IHTM is often confronted with such situations while performing consulting tests to identify antibodies to HFAs [45, 46]. At present, the major challenge for Polish blood transfusion service and IHTM is the implementation of molecular biology methods for i.e. low-frequency blood group antigens typing (LFA, the less frequent antithetic variants of HFAs), in order to identify donors with no HFAs.

The IHTM has presented the outcome of 17-year studies on testing antibodies to HFAs. This data illustrates what i.e. rare blood group donors should be at the disposal of blood transfusion centers in order to provide safe transfusions for immunized patients, pregnant women and their fetuses/newborns [46]. The types of rare blood donors are listed in the Ministry of Health Regulation of 06.02.2017 (Journal of Laws 2017.235).

As a part of the research funded by the IHTM, methodology based on real-time PCR was developed for identifying donors compatible with patients with anti-Rh51(MAR)-like antibodies. Genetic typing is the only method of determining homozygous Cw/Cw genotype in C+/Cw+ individuals [47]. The register of 7 Cw/Cw donors identified at that time required expansion and in 2019 the IHTM was once more in search for such donors for a different patient (4 new donors were identified).

The IHTM has also developed low-cost screening tests for genotyping the most frequent mutations responsible for lack of expression of common HFAs such as: Yt\(\alpha\), Kp\(b\), Di\(b\), Co\(a\), Lu\(b\), Kn\(a\), Lw\(a\), Vel, LAN [48]. A total of 948 donors were examined with the outcome of 6 donors with no Yt\(\alpha\) antigen and one LAN negative donor. Currently, studies have been expanded to include genotyping of antigens: Lu8/14, SC1/2, Jr\(a\) (2 most common mutations), LAN (subsequent mutations), P1/P2. It should be stressed that the latter antigen typing is not possible with the commercially available tests since none of these have the reagents for their identification.

The IHTM sees the necessity of establishing a National Registry of Rare Blood Group Donors operated within the currently developing e-blood system which is created in order to support the Polish Blood Transfusion Service. Conversion of data into digital (i.e. computer-readable) format will facilitate supervision over the register resources, ensure permanent access to rare blood group components (management of collection schedule, stocks of frozen components) and render the registry resources open to foreign registers.

7. Establishment of a registry of HPA and HNA typed donors for immunized patients

Molecular biology methods are the leading methods for HPA and HNA typing of patients suspected of alloimmunization as well as of blood donors; no commercial tests for phenotyping of these antigens are available (with the exception of HPA-1a antigen). Of particular importance is the establishment of registries of donors with typed HPA for diagnostic and transfusion purposes (Table 3) [7, 8, 49–52].

The IHTM has published a list of accessible HPA and/or HLA typed donors of platelet concentrates (PCs) together with the methods used for their identification [52] and points to the necessity of establishing a National Registry of Donors with marked HPA operated also by the e-blood system. The registry should be expanded to include more HPA-1a and HPA-5b negative donors due to the growing demand for this phenotype PCs dedicated primarily for fetal and neonatal transfusions in cases with recognized/suspected fetal/neonatal alloimmune thrombocytopenia (FNAIT) as result of feto-maternal incompatibility [53–56]. Donor-recipient genotype matching with regard to PCs may also apply to other HPA antigens, depending on the specificity of the anti-HPA antibodies. It may additionally require matching in HLA class I antigens [57].

As regards HNA antigens, the literature reports raise the need for HNA-3b/b genotype determination. Such donors should be tested for anti-HNA-3a antibodies, because they are the most common cause of Transfusion-Related Acute Lung
Injury (TRALI), induced by immunization with HNA antigens. It is even postulated that donors with anti-HNA-3a antibodies should be deferred from donating blood for clinical use [58, 59].

In summary, molecular biology methods are used in the IHTM reference laboratory for every-day consultations under subsidy for the activities of the public blood transfusion service (Article 25 of the Act on public blood transfusion service of 22 August 1997). The laboratory also depends on its own funds and grants for implementation and recommendation of methods that strengthen the safety of blood transfusion

References


