The burden of false RhD phenotyping of patients with RHD variants
Report of a case with RHD*10 DAU

WOLF D. KUHLMANN

MVZ für Laboratoriumsmedizin Koblenz-Mittlerhein, 56068 Koblenz
Labor – Diagnostik – Beratung, 56112 Lahnstein

Abstract
The Rh blood group is a polymorphic system with significant issues in blood transfusion. This holds especially true for Rhesus D antigen. Serologic RhD typing can be tricky due to the reason that monoclonal anti-D reagents may react with weak and partial D types in a variable manner. In a case of pregnancy with later proven RHD*10 (DAU) and categorized as partial D, we were stopped short of false RhD phenotyping because all serologic tests resulted in full strength reaction with several different anti-D reagents in different standard tests. At no time, agglutination signs for a weak or partial D variant were observed. The DAU cluster and other RHD variants are not prevailing in European population, but generally D variants may become more frequent with migrations and, thus, the possibility of false D antigen typing should be minded. In this context molecular RHD analysis will become increasingly required to secure the correct RhD status.

Key words

Introduction
Laboratories have policies for RhD typing as to detect and interpret the D-positive or the D-negative status of a patient. Despite serologic advances, discrepancies in Rh typing still occur due to genetic causes in the variability of D antigen expression leading to many different weak D and partial D phenotypes (ISBT 2019). Great variability is seen in RhD testing by use of monoclonal anti-D reagents (JUDD WJ et al. 2005, MOULDS MK 2006). In the case of weak D types or partial D, it is possible that serologic testing is not safe in the prevention of D antigen immunization (SANDLER SG et al. 2015).

The present study was induced by the case of a pregnant woman with suspicious Rhesus D antigen expression. RhD typing was discrepant in two laboratories. In one laboratory, the patient’s red blood cells (RBC) were serologically 0 RhD-positive ccDee and Kell negative, and no conspicuous features of the RhD antigen testing were found. In the other laboratory (notified about the African origin of the patient), the patient was RhD variant and the blood group documented as 0 RhD-negative ccddee, K negative; at the reverse side the patient was described “as donor Rhesus positive and as recipient Rhesus negative”. Hence, serologic RhD results differed significantly, the case deemed it right to be revised by serologic and molecular genetic methods.

Material and Methods

Blood group typing followed the guidelines for hemotherapy and immunohematology released by the German Medical Association (BUNDESÄRZTEKAMMER 2017).

Standard methods in serologic immunohematology are hemagglutination tests for which several assay designs exist:

- Direct agglutination (tube, slide, spot plate, microplate
- Microtube column agglutination (principle of agglutination and gel filtration)
- Microplate agglutination with automated work-up (autoanalyzer).

Agglutination in test tubes

Direct agglutination of erythrocytes with specific antibodies is done by detection of visible clumping in test tubes. For this approach, monoclonal antibodies of the IgM type were used without further aids.

Anti-D reagents for RhD typing:

- Immucor Anti-D (IgM + IgG): anti-D monoclonal IgM (TH28) and anti-D monoclonal IgG (MS26)
- Immucor Anti-D (Anti-D fast IgM): anti-D monoclonal IgM (D175-2)
- Immucor Anti-CDE (IgM + IgG): anti-C monoclonal IgM (MS24), anti-D monoclonal IgM (MS201), anti-E monoclonal IgM (MS80) and anti-D monoclonal IgG (MS26).

Column agglutination

The combined agglutination and centrifugation method according to the principle described by LAPIERRE Y et al. (1990) and RUMSEY DH and CIESIELSKI DJ (2000) was applied using the microtyping systems from Bio-Rad Laboratories and Ortho Clinical Diagnostics:

- Ortho BioVue System, ID-Cassette 707119 A, B, AB, D (DVI), D (DVI), ctrl with anti-D monoclonal IgM (D7B8) anti-D monoclonal IgM (RUM1)
- Bio-Rad ID-Micro Typing System, ID-Card 001344 A, B, D (DVI), D (DVI), ctrl, DAT with anti-D monoclonal IgM (LHM50/3 [LDM1], TH-28, RUM-1) and anti-D monoclonal IgM (LHM59/20 [LDM3], 175-2).

The Bio-Rad ID-Micro Typing System served for the determination of Rhesus subgroups and K antigen, two different clones of monoclonal antibodies for each antigen:

- ID-Card 002124 containing C, c, E, e, K, ctrl
- ID-Card 002224 containing C, c, E, e, K, ctrl.

Automated microplate system
Blood group typing with the Beckman Coulter (Olympus) PK7300 Automated Microplate System was carried out by Dr. BRILLAT (BwZKhrs Koblenz, Abt. XXII Transfusion Medicine). The method relies on the principle of agglutination in microplates and an automated work-up of all working steps with recording of the reactions by a CCD camera; the readings follow the chosen threshold settings for each reagent.

Patient samples were processed by two instruments with different reagents. The following anti-D reagents were used:

- Immucor Series 4 anti-D (Blend IgG und IgM) with anti-D monoclonal IgM (MS201) and anti-D monoclonal IgG (MS26)
- Immucor Series 5 anti-D (Blend IgG und IgM) with anti-D monoclonal IgM (TH28) and anti-D monoclonal IgG (MS26)
- Sifin anti-D IgM with anti-D monoclonal IgM (BS225).

**Other serologic assays**

The indirect antiglobulin test (COOMBS RR et al. 1945, IAT) at 37 °C (Bio-Rad LISS/Coombs Card 004014 and test erythrocytes ID-DiaCell I-II-II) is used for alloantibody screening.

Generally, samples which are negative with anti-D reagents are submitted to an IAT (test tube method or Bio-Rad column microtyping system) to detect weak D types and partial D (MUIRHEAD EE and JENNINGS ER 1964). Those cases and also, cases with differences in anti-D reactivity between two reagents are typical candidates which are forwarded to the Blood Transfusion Service of the German Red Cross (Bad Kreuznach) for molecular RHD typing.

The direct antiglobulin test (DAT, Bio-Rad LISS/Coombs Card 004014) is applied to detect sensitization of the patient’s erythrocytes. The DAT step is appropriate to avoid false positive D typing in IAT if the patient turns out to be positive in DAT.

**Controls**

Quality controls followed the legal regulations (BUNDESÄRZTEKAMMER 2014, 2017).

**RHD analysis**

The Blood Transfusion Service of the German Red Cross in Bad Kreuznach was contracted for RHD genotyping.

**Results**

The patient’s RBC typed with all serologic techniques as blood group 0 RhD positive, Rh subgroups ccee and K antigen negative. In several independent serologic controls, no conspicuous features of RhD could be observed. The ill-defined RhD status of the patient was finally the reason to perform molecular RHD genotyping with the result that the serologic result was indeed misleading.

RHD genotyping revealed the Rhesus D variant RHD*10 (DAU). This D variant applies to partial D in accordance with the International Society of Blood and Transfusion (ISBT 2019) and The Human Rhesus Base (RHEUSBASE 2018). D variants in general may cause alloimmunization, such patients should be classified Rhesus D-positive as blood donor and Rhesus D-negative as blood recipient in transfusion situations.

Table 1 shows the results of D antigen typing. Serologic agglutination strengths from 3+ to 4+ in immediate spin reflect the feature of a normal D protein and is evaluated as RhD-positive. From weak D types and D variants one would expect weak or even negative agglutinations. Results are reproducible with either of the employed methods and irrespective of the assay type; the agglutination behavior is indicative for a conventional D antigen.
### Tabelle 1. Results of RhD antigen tests with different anti-D reagents

<table>
<thead>
<tr>
<th>Anti-D reagents source and clones</th>
<th>Test tube agglutination *</th>
<th>Bio-Rad gel matrix **</th>
<th>Ortho BioVue glass beads **</th>
<th>Microplate analyzer ***</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immucor</td>
<td>+++ to ++++</td>
<td>N/A ****</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Anti-D (IgM + IgG) monoclonal IgM and monoclonal IgG TH28, MS26</td>
<td>++++</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Anti-D (fast IgM) monoclonal IgM D175-2</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Anti-CDE (IgM+IgG) monoclonal IgM and monoclonal IgG MS24, MS201, MS80, MS26</td>
<td>++++</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Bio-Rad ID-System</td>
<td>N/A</td>
<td>+++</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Anti-D monoclonal IgM LHM50/3, TH-28, RUM-1</td>
<td></td>
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<tr>
<td>Anti D monoclonal IgM LHM59/20, 175-2</td>
<td></td>
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<tr>
<td>Ortho Vision/BioVue</td>
<td>N/A</td>
<td>N/A</td>
<td>++++</td>
<td>N/A</td>
</tr>
<tr>
<td>Anti-D monoclonal IgM D7B8</td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Anti-D monoclonal IgM RUM1</td>
<td></td>
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</tr>
<tr>
<td>Immucor Series 4 and 5</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>positive *****</td>
</tr>
<tr>
<td>Anti-D monoclonal IgG, IgM a) MS26, MS201 b) MS26, TH28</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sifin Anti-D monoclonal IgM BS225</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>positive *****</td>
</tr>
</tbody>
</table>

* Agglutination strengths of 3+ and 4+ reflecting RhD-positive status
** Column agglutination method using Bio-Rad and Ortho BioVue card systems, respectively
*** Microplate analyzer Olympus PK7300 with instrument setting according to the manufacturer
**** N/A (not applicable), reagents not in use for this assay type
***** Agglutination value (positive) according to the instrument settings

Further results: control reactions were regular. No signs of autoagglutination or sensitization of the patient’s erythrocytes were observed. Tests for the presence of alloantibodies were negative.
Discussion

In our case study, the patient sample reacted in all serologic tests in the same manner. Due to the strong reactivity of the different anti-D reagents, the patient’s RhD feature was assessed being RhD-positive, and no doubts were left about the patient’s blood group 0 RhD-positive. All results were discussed with the Blood Transfusion Service of the German Red Cross (Bad Kreuznach) and a molecular analysis of the RHD gene was phased.

**RhD antigen and RHD gene.** Molecular analyses of the RHD gene revealed RHD*10 (DAU), this DAU variant is categorized as partial D (RhesusBase 2018). The result was at first astonishing from all the above findings with standard serology. D variants are quite unusual in clear-cut D serology. Several studies with monoclonal antibodies, however, have shown that serologic detection of weak D or partial D types is not easy at all because of variabilities in serologic test settings (JONES J et al. 1995a and 1995b, JUDD WJ et al. 2005, Denomme GA et al. 2008, Lai M et al. 2009, Sandler SG et al. 2017).

**DAU** variants may show variable strength of reaction with anti-D reagents (Wagner FF et al. 2002, Duncan JA et al. 2017). Yet, surprisingly, in the present case of RHD*10 (DAU) full strength reactions occurred with monoclonal anti-D antibodies. RHD variants derived from the DAU allele cluster (cluster of linked homologous genes) occur frequently in Africa, while they are rare in Europe. The present case highlights the limitations of standard serologic procedures. In general, the possibility of D antigen immunization as in other weak D phenotypes or partial D variants must be considered in transfusion and in pregnancy (Wagner FF et al. 2000, Wagner FF et al. 2002, Rizzo C et al. 2012, McBain RD et al. 2015, Srivastava K et al. 2016). The incidence of D immunization, however, is difficult to predict. For patients with DAU alleles, the following Rhesus D classification for medical issues such as blood transfusion or anti-D prophylaxis in pregnancy is advised:
mutations of the Rhesus DAU from two patients of African ancestry with completely expressed D antigen. This complies with observations of The DAU cluster weak D types SW serologic tests it is known that r weak D or partial D which cannot be defined by positive or SW molecular means. D antigen, especially with respect to qualitative differences from a normal RhD antigen. This complies with observations of The DAU cluster weak D types and D variants compared with the strong reactions of fully expressed RhD antigens (e.g. 1+ or 2+) when compared with erythrocytes of normally expressed D antigen (JONES J et al. 1995a and 1995b, DENOMME GA et al. 2005, JENKINS CM et al. 2005, WESTHOFF CM, 2005, DENOMME GA et al. 2008, LAI M et al. 2009, FLEGEL WA 2011, SANDLER SG et al. 2015). Monoclonal anti-D reagents can never distinguish between different weak D types and D variants. It remains to accept the inconsistency of serologic assays.

The presented results from RhD antigen typing are an example for risks of false conclusions from serologic testing. According to the current hemotherapy guideline, molecular biological methods are just indicated to clarify ambiguity in ABO, RhD or other blood group serology. However, what about cases with D variants that do not show serologic abnormalities and where it much later turns out that RhD variants is present? How to reconcile with the possibility of serologic pitfalls? No schemes are specified how to anticipate critical situations.

Serologic reagents. Generally, discrepancies are inherent to serologic methods. They are by no means to exclude, even not by changing the composition of reagents (LAI M et al. 2009). Anti-D reagents contain various monoclonal antibodies, various additives, and among other things, proteins in different concentrations to act as agglutination enhancers (MOULDS MK 2006). Technically, this will not corrupt agglutination tests and as a rule, weaker reactions with D variants compared with the strong reactions of fully expressed RhD antigens are to be expected. The main problem with smart changes within the D antigen can only be solved by molecular means.

Weak D types and D variant. Apart from individuals being serologically clear-cut RhD-positive or clear-cut RhD-negative, there exist a grey area plenty of D variants categorized as weak D or partial D which cannot be defined by any serologic assay. From experimental data it is known that red blood cells with weak D types and D variant usually react weaker in serologic tests (e.g. 1+ or 2+) when compared with erythrocytes of normally expressed D antigen (JONES J et al. 1995a and 1995b, DENOMME GA et al. 2005, JENKINS CM et al. 2005, WESTHOFF CM, 2005, DENOMME GA et al. 2008, LAI M et al. 2009, FLEGEL WA 2011, SANDLER SG et al. 2015). Monoclonal anti-D reagents can never distinguish between different weak D types and D variants. It remains to accept the inconsistency of serologic assays.

The DAU cluster. In our described case, serologic paradox occurred because RhD mimicked a completely expressed D antigen This complies with observations of DUNCAN JA et al. (2017) from two patients of African ancestry with RHD*DAU5 allele.

Rhesus DAU is a cluster of at least 18 alleles with a cDe haplotype for which one or more mutations of the RHD gene are characteristic (WAGNER FF et al. 2002, WAGNER FF and
FLEGEL WA 2014, RHEUSBASE 2018, OMIM 2019). The D variant of our patient belongs to RHD10.00 (DAU-0), probably with a single missense mutation at 1136C>T (T379M) whereas all other alleles listed in The Rhesus Base (RHEUS BASE 2018) have multiple missense mutations. The phenotype of DAU-0 is designated as D-positive (and apparently normal) but categorized as partial D. This could explain the patient’s reactivity in serologic D antigen testing as RhD-positive exhibiting no signs of partial D.

Obviously, the detection of weak D or partial D can be a difficult task or is even impossible by serologic typing. Mismatching and pitfalls in blood group serology may occur more often than thought. Genetic and ethnic factors and other reasons for gene conversion (RHD, RHCE) or mutations highlight limits of serology. This is a challenge for guideline-compliant diagnostics. Studies of RHD alleles in D-negative and D-positive Europeans gave evidence that the variety of RHD alleles is probably larger than anticipated (WAGNER FF et al. 2001, CHEN Q and FLEGEL WA 2005). Apart from certain genetic-ethnic factors, the overall diversity of RHD genes will have an impact on diagnostic strategy. One really would welcome compelling reference and guidelines addressed to serologic failures in RhD testing.

Information about geographical and ethnic origin of patients can be helpful in the diagnostic design (FLEGEL WA 2006, FLEGEL WA 2007a and 2007b, FLEGEL WA 2011, FLEGEL WA et al. 2014). In the prevention of RhD antigen immunization in situations such as transfusion or pregnancy one must reconcile the failure of serology. The question is open to define clear-cut conditions under which genetic analysis of RHD is needed.

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References


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