Rhesus D variants in pregnant women: results of a one-year observation period

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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. RHD variants such as weak D type 1, type 2 and type 3 are quite common in the European population while other weak D types and partial D variants are observed rarely. This, however, is changing due to migration, and the possibility of false D antigen typing in cases of blood transfusion and pregnancy should be minded. In this context molecular RHD analysis will become increasingly needed to secure the correct RhD status.

Key words

Blood group serology – D antigen – D variant –weak D –partial D – ethnic background – molecular diagnostics

Introduction

Rhesus D antigen of the RH blood group belongs to the most important blood group antigens determined by a protein. In Europeans, antigen D on red blood cells (RBC) is closely linked to the so-called standard *RHD* allele (ISBT 2019) and absence of antigen D correlates well with deletion of the *RHD* gene; for ISBT blood group terminology see DANIELS GL et al. (2004). About 1% of Europeans carry aberrant *RHD* alleles (WAGNER FF et al. 1995, ROUBINET F et al. 1996, WAGNER FF et al. 1999, WAGNER FF et al. 2001, FLEGEL WA and WAGNER FF 2002). Aberrant *RHD* alleles encode Rh D variants which are the cause of problems in serologic diagnostics and which hold the intrinsic properties of alloimmunization.

The distinction of weak D types and partial D from normal D antigen (D-positive) and D-negative is of clinical relevance due to possible alloimmunization in situations such as transfusion and pregnancy. Studies have shown that serologic procedures can be ambiguous in the detection of clinically relevant RhD variants. The possibility of variable agglutination by anti-D serum was already observed in 1946 (STRATTON F 1946) and D variants were named D^u. Such serologic properties are unfavorable to determine a correct RhD status because of inherent false typing results in patients with weak D or partial D types. Hence, serologic methods were improved and, more importantly, molecular RHD genotyping was introduced with significant progress in diagnostics (JONES J et al. 1995, TIPPETT P et al. 1996, WAGNER FF et al. 2000, LEGLER TJ et al. 2001, PERCO P et al. 2003, DENOMME GA et al. 2005, JENKINS CM et al. 2005, JUDD WJ et al. 2005, WESTHOFF CM 2005, MOULDS MK 2006, DENOMME GA et al. 2008, FLEGEL WA et al. 2009, LAI M et al. 2009, FLEGEL WA 2011, DANIELS G 2013, SANDLER SG et al. 2015, SANDLER SG et al. 2017). Many of the technical

RHD setups are adapted to samples from European people that are not always the right ones to detect rare alleles and genetic variants in general (LEGLER TJ et al. 2001, WAGNER FF et al. 2001, GASSNER C et al. 2005).

Screening of RhD-positive blood donors in southwestern Germany by PCR-SSP has shown that up to 12 single-nucleotide polymorphisms can be found that are representative for the most frequent RHD alleles in Europeans (CHEN Q and FLEGEL WA 2005). From this and other studies one can conclude that the variety of *RHD* alleles may be larger than anticipated (AVENT ND et al. 1997, WAGNER FF et al. 2001, GASSNER C et al. 2005). Furthermore, aberrant *RHD* alleles are not a random variable because significant ethnic variabilities in weak D and partial D types occur (GROOTKERK-TAX MG et al. 2005). Serologic testing is of limited choice to distinguish between RhD-positive and RhD-negative or weak D and partial D. Also, patients with partial D phenotype may be falsely typed as D-negative. Serology is not safe at all in the prevention of D antigen immunization.

In the last years we noted an increase of inconclusive reactions in RhD antigen serology. We report here the observations from a study period of 12 months with specimens from pregnant women which we obtained on occasion of maternity care. Major interest was safe classification of Rhesus D either as RhD-negative or RhD-positive and the possible detection of weak D and partial D variants for the prevention of anti-D-alloimmunization.

Material and Methods

Patients. Specimens from pregnant women were studied for the occurrence of Rhesus weak D types and partial D variants during an observation period of 12 months (01.04.2019 to 31.03.2010). The cohort included a collection of 3055 blood samples. The cohort was quite multi-ethnic. A large part of the pregnant women was from Middle East, another part was from different European territories including Germany, and a minor part of pregnant women was from Africa.

Samples. Patients attended medical practices for maternity prevention. Blood samples were taken by gynaecologists and sent in for routine prenatal testing covering AB0 blood group and Rhesus D phenotyping and the search for alloantibodies; no serologic data were known so far. All examinations were in compliance with the governmental rules of maternity prevention (G-BA Mutterschafts-Richtlinien Mu-RL, 2020).

Reagents. Generally, certified in vitro reagents were applied. AB0 traits were specified by monoclonal antibodies and results being secured by testing of the corresponding blood isoagglutinins. Rhesus D antigen was determined by two different anti-D monoclonal IgM antibodies. A panel of defined test RBCs was used for alloantibody screening. In case of conspicuous features of serologic tests, appropriate diagnostic assessments followed as postulated by governmental guidlines (BUNDESÄRZTEKAMMER 2017).

Routine blood group testing

The gel column technology in connection with the automated microtyping system from Ortho Clinical Diagnostics (Ortho Vision Analyzer and Ortho BioVue ID-Cassette 707119 A, B, AB, [D^{VI-}], [D^{VI-}] and ctrl) was used in routine. RhD antigen was determined by monoclonal IgM anti-D preparations (D7B8 and RUM1).

Evaluation of agglutination

Agglutination reactions are graded either as strong positive, positive, weak positive or as negative (grading system from 4+ to 0), depending on the size of the RBC band at the top of the gel column; negative reactions represent the absence of a band of RBCs at the top of the gel column and RBCs deposit at the bottom of the microtube. Specimens carrying the antigen D with defined normal strength always give a strong positive agglutination reaction (4+ in the grading system).

From the selection of monoclonal anti-D reagents one might expect to have some dissimilar specificities for the likelihood to pick out D variants (DENOMME GA et al. 2005). It is common practice that samples with agglutination reactions of 3+ strength (instead of 4+) or even less strengths are rated as suspect (for instance weak D or partial D). Those cases were forwarded to the Red Cross Transfusion Service (Institute Bad Kreuznach) for *RHD* genotyping by a molecular PCR method.

Direct antiglobulin test (DAT)
The direct antiglobulin test (DAT, Bio-Rad LISS/Coombs Card 004014) is applied to detect *in vivo* sensitization of patient's erythrocytes. DAT is an appropriate step to exclude false positive indirect antiglobulin testing (IAT) in the search for a weak RhD antigen.

RhD antigen agglutination in test tube

This approach is a supplement in antigen D testing when samples turn-out as RhD-negative in serologic blood group testing. RBC agglutination is done in test tubes by a panel of anti-D reagents. Anti-D reagents used:

- 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).

Sensitization of erythrocytes may occur in cases of weak D or partial D. The indirect antiglobulin test (IAT) is a useful method for their detection (MUIRHEAD EE and JENNINGS ER 1964, JENKINS CM et al. 2005, DANIELS G 2013, SANDLER SG et al. 2014).

Indirect antiglobulin test (IAT)

The indirect antiglobulin test in LISS/Coombs milieu (COOMBS RR et al. 1945) is done with a column microtyping system using Bio-Rad LISS/Coombs Cards. Positive IAT reactions are indicative of weak D or D variant types in the samples which had to be forwarded to the Red Cross Blood Transfusion Service (Institute Bad Kreuznach) for molecular *RHD* typing.

Other serologic assays

Alloantibody screening is done by indirect antiglobulin tests using Bio-Rad LISS/Coombs Cards and ID-DiaCell I-II-II test erythrocytes (Bio-Rad).

Controls

Quality controls comply with legal regulations (BUNDESÄRZTEKAMMER 2017, 2019).

RHD genotyping

Genotyping was done by the Red Cross Blood Transfusion Service (Institute Bad Kreuznach) using the PCR-SSP method.

Results

From the study of 3055 pregnant women we detected 22 cases (about 0.7 %) with inconclusive RhD antigen results by routine serology. Validity was in question when monoclonal anti-D reagents reacted differently, e.g., one antibody resulted with a 3+ and the other antibody with a 4+ reaction in the assays. Such small agglutination differences were reliably documented by the optical system of the microtyping system (Ortho Vision Analyzer) and rated by us to be important for further examination. More pronounced agglutination differences (e.g. 2+ or 1+ reactions) were not seen in our test samples. Additional methods with alternative test designs were not able to give more information with regard to agglutination quality than the gel column technique.

The 22 cases with inconclusive RhD antigen reaction were selected for subsequent *RHD* genotyping. Specimens from a pregnant African woman were included in this study although no serologic discrepancies were observed but for the reason of a varying report from another laboratory.

Genotypic analysis of the 22 cases revealed 18 women as weak D carriers. The majority (10 cases) were of weak D type 1, four cases were of weak D type 2 and three cases of weak D type 3. Then, one specimen was reported as weak D type 31.

Four women were classified as partial RHD. The most interesting partial D case, however, was that of a serologic normal D antigen which later on proved to be partial D (DAU) by molecular typing, i.e. RHD*10 (RHD*DAU0). Figure 1 shows the serologic blood group typing of this special case. The relevant data from all *RHD* genotyped cases are summarized in three tables (Tab. 1 to 3).

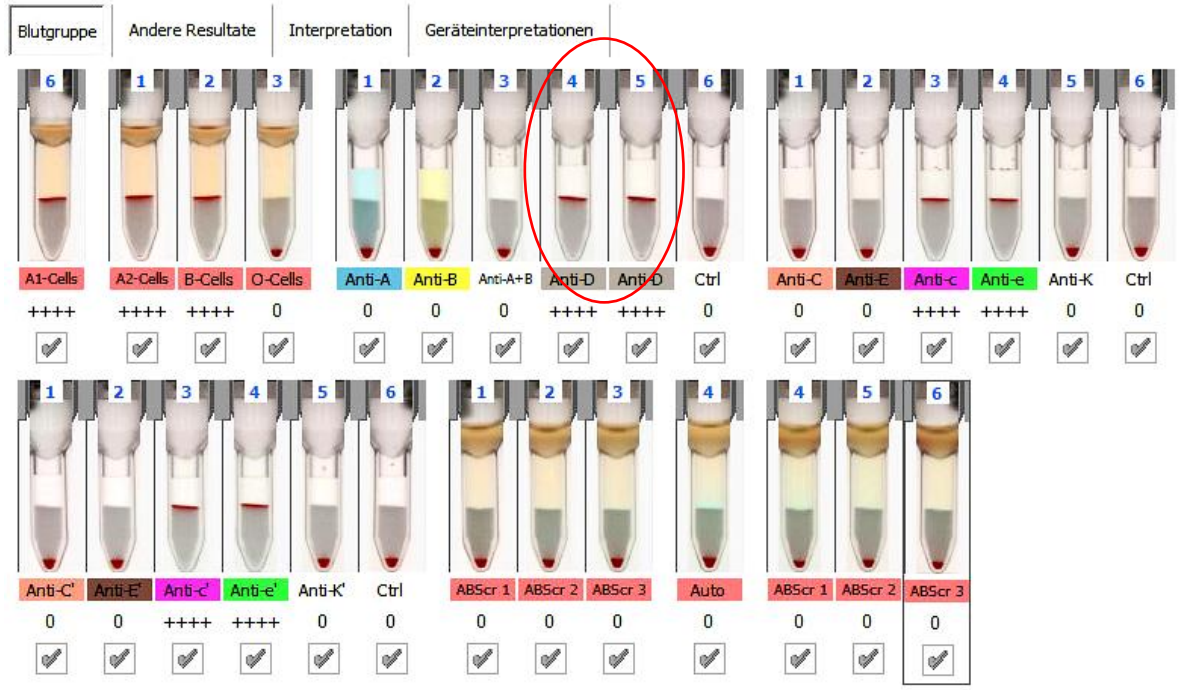

Fig. 1: Blood group serology of the patient with variant RHD*10 (DAU) using Ortho BioVue ID-Cassette 707119. Note anti-D microcolumns with 4+ reactions by each of the monoclonal antibodies and reflecting apparently normal RhD antigen

Tab. 1: RhD typing: serologic RhD analysis and results from *RHD* genotyping

Cases (*n*)	**RhD antigen** [1]	**ISBT group** [2]	**Designation**	**ISBT name**	**Cluster**
10	3+/4+	weak D	weak D type 1	RHD*01W.1	Eurasian D cluster
4	3+/4+	weak D	weak D type 2	RHD*01W.2	Eurasian D cluster
3	3+/4+	weak D	weak D type 3	RHD*01W.3	Eurasian D cluster
1	3+/4+	weak D	weak D type 31	RHD*01W.31	Eurasian D cluster
1	3+/4+	partial RHD	partial D (RH33) [3]	DHAR (004.033)	Eurasian D cluster
1	3+/4+	partial RHD	partial D (DAR)	RHD*09.01	weak D type 4 cluster
1	3+/4+	partial RHD	partial D (DAR2)	RHD*09.02	weak D type 4 cluster
1	4+/4+ [4]	partial RHD	partial D (DAU)	RHD*10	DAU cluster
Total number of patients (*n* = 22) with Rhesus weak D types or partial D					

[1] Serologic RhD antigen agglutination in Ortho BioVue Cassettes with two monoclonal antibodies, each in a separate microcolumn: clone D7B8 and clone RUM1. Reaction gradings: strong positive (4+), positive (3+), weak positive (2+/1+) or negative (0)

[2] Names and nomenclature derived from terminology and data entries in RHESUSBASE (2018) and ISBT (2019)

[3] Partial D phenotype RH33 (R_0Har, Rh:33), ISBT symbol (number) RH33 (004.033). R_0Har is associated with an *RHCE-D(5)-CE* hybrid allele and first described by BECKERS EA et al. (1996a, 1996b). Description in RHESUSBASE (2018) as DHAR, antigens expressed are Rh33 and Rh50 (WAGNER FF and FLEGEL WA 2004)

[4] Apparently normal D antigen in serology. Partial D (DAU) uncovered by *RHD* genotyping; data from RHESUSBASE (2018) see Tab. 2

Tab. 2: Partial D (DAU), *RHD*10 (DAU)* data derived from RHESUSBASE (2018) http://www.rhesusbase.info/RHDDAU-0.htm

Change from standard allele	**ISBT allele designation** [1]	**Amino acid change**	**ISBT group**	**Phenotype grouping**	**Haplotype (typical)**
1136C > T (T379M)	RHD*10 RHD*DAU0	Thr to Met at codon 379	Partial RHD	D positive (apparently normal) partial D	cDe

[1] *DAU* alleles constitute a cluster of alleles. They share a 1136C > T single nucleotide polymorphism causing a T379M substitution (WAGNER FF et al. 2002). The single polymorphism is only found in *RHD*DAU0* while other *DAU* alleles have one or 2 additional substitutions. This is noteworthy because other alleles than *RHD*DAU0* result in different serologic RhD patterns

Tab. 3: RHD genotyping and recommendations for pregnant women and transfusion situations

Weak D (*n* = 18)	**ISBT name**	**Designation**	**D antigen status report**
Patients *n* = 10	RHD*01W.1	weak D type 1	Patient as blood donor: RhD-positive Patient as blood recipient: RhD-positive RhIG prophylaxis: **No**
Patients *n* = 4	RHD*01W.2	weak D type 2	Patient as blood donor: RhD-positive Patient as blood recipient: RhD-positive RhIG prophylaxis: **No**
Patients *n* = 3	RHD*01W.3	weak D type 3	Patient as blood donor: RhD-positive Patient as blood recipient: RhD-positive RhIG prophylaxis: **No**
Patients *n* = 1	RHD*01W.31	weak D type 31	Patient as blood donor: RhD-positive Patient as blood recipient: RhD-negative RhIG prophylaxis: **Yes**
Total number of patients with Rhesus weak D types (*n* = 18)			
Partial D (*n* = 4)	**ISBT name**	**Designation**	**D antigen status report**
Patients *n* = 1 Patients *n* = 1 Patients *n* = 1 Patients *n* = 1	DHAR (004.033) RHD*09.01 RHD*09.02 RHD*10	partial D (RH33) partial D (DAR) partial D (DAR2) partial D (DAU)	All patients with partial D: (a) as blood donor RhD-positive (b) as blood recipient RhD-negative (c) RhIG prophylaxis: **Yes**
Total number of patients with Rhesus partial D variants (*n* = 4)			

Discussion

Patients with inconclusive RhD antigen serology gave evidence for a variety of weak D types and partial D variants when further examined by *RHD* genotyping. Studies of European persons as well as long-term experience with blood donors in services such as the DRK (German Red Cross) have shown that within the European population the variety of *RHD* alleles might be higher than usually thought (WAGNER FF et al. 2001, CHEN Q and FLEGEL WA 2005, FLEGEL WA et al. 2009). With migration one can expect still more D variants. Knowledge of ethnic backgrounds can be helpful in the diagnostic design (FLEGEL WA 2006, FLEGEL WA 2007, FLEGEL WA 2011, FLEGEL WA et al. 2014). Serologic limitations will be only overcome by integration of *RHD* genotyping into the diagnostic process with the need of a continued method adaptation to prevalent alleles (FLEGEL WA et al. 2009).

In spite of the small cohort, our collection was quite multi-ethnic. A large part of the pregnant women were Middle East refugees, others originated from different European territories and some came from Africa. One can assume that RhD variants might rise from an actual level of below 1.0 % to higher values in the future. About 90 % of European RhD variants are of weak D type 1, 2 or 3 and can be regarded as being RhD-positive both as blood donor and as blood receiver. In these cases, pregnant women do not need RhIG prophylaxis. In contrary, other cases of weak D and especially D partial variants are throughout matched RhD-positive both as blood donor and as blood receiver with the need of RhIg prophylaxis in pregnancy. We can expect increasing numbers of allele varieties by migration and a greater variety of weak D types and partial D than before, and hitherto rare D variants have to be respected in diagnostic and transfusion situations.

Antigen frequencies differ in Europe, Africa and other territories (GROOTKERK-TAX MG et al. 2005, AVENT ND 2005, WAGNER FF et al. 2005). From Africa we know that aberrant RHD alleles are widespread, for example with *DAU-0* the most prevalent one of about 19% in West Africa (WAGNER FF et al. 2003). The problem of *DAU* variants is that they show variable strength of reaction with anti-D reagents (WAGNER FF et al. 2002, DUNCAN JA et al. 2017). Our present case, a pregnant woman with RHD*10 (DAU) and full strength reactions with monoclonal anti-D antibodies highlights the limitations of standard diagnostic procedures.

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, SRIVASTAVA K et al. 2016, RHESUSBASE 2018, OMIM 2019). The D variant of patients belonging to *RHD10.00* (DAU-0) are characterized by a single missense mutation at 1136C>T (T379M) whereas all other alleles listed in *The Rhesus Base* (RHESUS 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 explains the patient's reactivity in D antigen testing as RhD-positive showing no signs of partial D.

The term "serologic weak D phenotype" does not reflect the relevant clinical properties of the D antigen, neither by qualitative nor by quantitative differences from a normal RhD antigen. Changes in D epitopes are the main reason for the induction of transfusion related anti-D antibodies. The many reasons for gene conversion (*RHD*, *RHCE*) or mutations are limits for serology. The overall diversity of *RHD* genes will have an impact on diagnostic strategy. Compelling reference and guidelines addressed to serologic failures in RhD testing are welcome. It is known from many studies that *RHD* typing is one of the best ways to define the RHD state (TIPPETT P et al. 1996, AVENT ND and REID ME 2000, DOMEN RE 2000, WAGNER FF et al. 2000, LEGLER TJ et al. 2001, LURIE S et al. 2001, DENOMME GA et al. 2005, FLEGEL WA 2006, FLEGEL WA et al. 2007, FLEGEL WA et al. 2009, PHAM BN et al. 2011, FLEGEL WA and DENOMME GA 2012, DANIELS G 2013, SANDLER SG et al. 2014, SANDLER SG et al. 2015, SANDLER SG et al. 2017, LUKACEVIC KRISTIC J et al. 2018). We should remind, however, the possible occurrence of RHD among apparently RhD-negative persons (GASSNER C et al. 2005).

Classical serologic tests remain important but molecular techniques are more and more required to assure RHD blood group results. Nevertheless, we must recognize limitations of PCR methods. Many of the test setups are adapted to samples from European people. Yet, these methods must not be under all circumstances the right ones to detect rare alleles (LEGLER TJ et al. 2001, WAGNER FF et al. 2001, GASSNER C et al. 2005). Failure in genotyping will occur by undescribed mutations (false-positive and false-negative results).

Also, diseases such as hematologic malignancies (CHÉRIF-ZAHAR B et al. 1998) causing blood group chimerism or other reasons of somatic mutations may lead to alteration in blood group phenotypes. The *European Blood Directive* and the *BloodGen Project* have addressed the need for the development of appropriate concepts and technology (FABER JC 2004, AVENT ND et al. 2007).

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