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Application of a Multivariant, Caucasian-Specific, Genotyped Donor Panel for Performance Validation of MDmulticard®, ID-System®, and Scangel® RhD/ABO Serotyping

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Key Words
ABO blood group · Blood groups · DNA · Genotyping · Kell determination · Molecular blood group typing · Red blood cell antigens · Rh phenotyping · Serological typing

Summary
Background: Validations of routinely used serological typing methods require intense performance evaluations typically including large numbers of samples before routine application. However, such evaluations could be improved considering information about the frequency of standard blood groups and their variants. **Methods:** Using *RHD* and *ABO* population genetic data, a Caucasian-specific donor panel was compiled for a performance comparison of the three RhD and ABO serological typing methods MDmulticard (Medion Diagnostics), ID-System (DiaMed) and ScanGel (Bio-Rad). The final test panel included standard and variant *RHD* and *ABO* genotypes, e.g. RhD categories, partial and weak RhDs, RhD DELs, and *ABO* samples, mainly to interpret weak serological reactivity for blood group A specificity. All samples were from individuals recorded in our local DNA blood group typing database. **Results:** For 'standard' blood groups, results of performance were clearly interpretable for all three serological methods compared. However, when focusing on specific variant phenotypes, pronounced differences in reaction strengths and specificities were observed between them. **Conclusions:** A genetically and ethnically predefined donor test panel consisting of 93 individual samples only, delivered highly significant results for serological performance comparisons. Such small panels offer impressive representative powers, higher as such based on statistical chances and large numbers only.

§Retired.

Schlüsselwörter
AB0-Blutgruppe · Blutgruppe · DNA · Genotypisierung · Kell-Bestimmung · Molekulare Blutgruppentypisierung · Erythrozytenantigene · Rh-Phänotypisierung · Serologische Typisierung

Zusammenfassung
Hintergrund: Die Validierung von routinemäßig genutzten serologischen Typisierungsmethoden erfordert intensive Leistungsevaluierungen, die vor dem Routineeinsatz in der Regel große Mengen von Proben einschließen. Solche Evaluierungen können aber dadurch optimiert werden, dass Informationen über die Häufigkeit von Standardblutgruppen und ihrer Varianten berücksichtigt werden. **Methoden:** Unter Verwendung von populationsgenetischen Daten zu *RHD* und *AB0* wurde ein europäischstämmiges Donorpanel zusammengestellt, um die Leistungsfähigkeit von drei serologischen RhD- und AB0-Typisierungsmethoden, MDmulticard (Medion Diagnostics), ID-System (DiaMed) und ScanGel (Bio-Rad), zu vergleichen. Das endgültige Testpanel beinhaltete Standard- und variante *RHD*- und *AB0*-Genotypen, z.B. RhD-Kategorien, partielle und schwache RhDs, RhD DELs und AB0-Proben – hauptsächlich um die schwache serologische Reaktivität für die Blutgruppe-A-Spezifität zu beurteilen. Alle Proben stammten von Personen, die in unserer lokalen DNA-Blutgruppen-Typisierungsdatenbank erfasst sind. **Ergebnisse:** Für die Standardblutgruppen waren die Leistungsergebnisse für alle drei in den Vergleich eingeschlossenen serologischen Methoden eindeutig interpretierbar. Bei Betrachtung spezieller varianter Phänotypen wurden allerdings deutliche Unterschiede hinsichtlich der Reaktionsstärke und der Spezifität der drei Methoden deutlich. **Schlussfolgerung:** Ein genetisch und ethnisch vordefiniertes Donor-Testpanel, das aus lediglich 93 individuellen Proben bestand, liefert hochsignifikante Ergebnisse für den Leistungsvergleich serologischer Verfahren. Solche kleinen Panele verfügen über eine beeindruckende repräsentative Aussagekraft – größer als die, die allein auf statistischen Veränderungen und hohen Zahlen beruht.

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Introduction

Validations of routinely used serological typing reagents and methods usually require intense performance evaluations not only since the advent of Conformité Européenne (CE) labeled test kits [1]. Nowadays, common technical specifications for in vitro diagnostic devices define mandatory sample numbers for the performance evaluation of new test kits, which may reach 3,000 single testings for new anti-D, or anti-A, anti-B and anti-AB reagents [2]. Exigencies for the inclusion of rare phenotypes exist only for the detection of RhD with a rather general requirement that 'weak Ds' should be included in the test panel at a percentage greater than 2% of all Rhesus-positives investigated.

The above mentioned large sample numbers have been found appropriate for the purpose of validation of monoclonal antibodies and deduced typing kits for serological blood group typing. Additionally, these sample numbers may also be understood as precaution for delivering typing reagents not only capable of recognizing regular blood group antigens but also to reliably identify their infrequent and originally poorly defined variants by statistical chance, e.g. weakly expressed ABO or RhD antigens. However, validation of a test system with a large number of samples alone does not necessarily reflect the population of interest appropriately. Antigen variants with frequencies slightly lower than those to be expected in the investigated validation panel size would not be considered for evaluation, but almost certainly encountered on a regular basis in routine application of the respective typing systems later on.

Since the last decade of the 20th century, knowledge about human blood groups has been refined considerably by genetic findings, initiated by the first descriptions of *ABO* and *RHD* genetics in 1990 [3] and 1991 [4], respectively. These genetic findings offered unprecedented information with respect to the massive genetic polymorphism and definition of variant phenotypes. Additionally, the high definition of blood group phenotypes on their genetic basis also delivered very reliable frequency and geographical distribution – 'population genetic' – data, previously inaccessible to classical blood group serology.

The above described development is exemplified best by the elucidation of weakly expressed D antigens, originally termed 'D^u' and described by Stratton in 1946 [5]. Whilst blood group experts suspected this group of D^us to be heterogeneous, still serological methodology failed to define this group into clearly distinguishable subgroups. It was in 1999, when Wagner et al. [6] described the molecular basis of weak D phenotypes, hereby opening the way for the unambiguous definition and typing of every single 'D^u' sample. Shortly thereafter an impressive number of articles described the frequency of weak *RHD* alleles found in sample collections of phenotypically weak Ds analyzed in a variety of populations [7–9].

Considering these findings in the light of performance evaluation of serological test reagents and kits, a logical consequence unfolds: there is a need for well compiled test panels, representative of populations and discriminative in their composition. This requirement is achieved ideally by using genotyped donor panels considering the relative occurrence of single phenotypes included. In order to compare performance between MDmulticard (Medion Diagnostics), ID-System (DiaMed) and ScanGel (Bio-Rad) D and ABO serotyping methods, we focused mainly on *RHD*, used respective population genetic data, established a test panel, performed the typing, and analyzed the results.

Material and Methods

Configuration of the Test Panel for RhD and RhCE

With respect to RhCE, all independent C and E regular phenotypes were considered in RhD-negative and RhD-positive individuals. That means, that cc, Cc and CC as well as ee, Ee and EE individuals were each collected from RhD-negative and RhD-positive donors (n = 21). Additionally, variant RhDs, which are RhD categories and other partial RhDs, weak RhDs, RhDs only detectable by adsorption-elution techniques (RhD DELs), and RhD-negatives with a certain genetic background, were included in our genotyped donor test panel. Weak D red cells are considered to have all epitopes of D expressed weakly. Partial D red cells are qualitative variants, most times also expressed weakly, and including RhD categories as nomenclaturilly distinct group, historically described first by Tippett and Sanger [10–12]. All partial RhDs may develop anti-D upon confrontation with RhD after transfusion or pregnancy [10]. RhDs DELs show the lowest number of RhD molecules per erythrocyte, regularly interpreted as RhD-negative by routine serological methods [13].

Data of our local database, representing more than 2,000 single blood group DNA typing records of the last 12 years, were used and supplemented by appropriate reports to define potential *RHD* alleles of interest. Our local database is representative of the Western part of Austria named Tyrol with its population of approximately 700,000 inhabitants. Additionally, several hundred samples submitted from all over Austria are included in our database.

RhD category II was not encountered, and RhD category III was represented by one case of type IIIc only, although said to be frequent in the Caucasian population [14]. One sample of RhD category IV type 4 was included in the test panel although no reliable frequency data were available. D category V seems to be based on a highly polymorphic genetic and geographic background, but only one sample was present in our records und inaccessible for our test panel [14]. RhD category VI is known to be encoded by 4 different alleles, of which type 1 was found to have an allele frequency of 0.00076 with a resulting phenotype frequency (that is the situation where a *RHD* category VI type 1 allele is co-inherited with a *RHD*-negative haplotype) of 0.001339, or 1 in 1,494 individuals of our local population [7]. *RHD* category VI type 2 is present, but types 3 and 4 are not present in our database. No direct frequency estimates could be obtained for *RHD* category VI type 2 nor for the included *RHCE-D(5)-E* hybrid allele *DHAR* (Rh33) and *RHD* DFR type 1. Several RhD category VII and partial RhD DNB samples were present in our database. Comparing the number of observations and the known frequency for RhD category VI type 1 with the number of observation for RhD category VII and partial RhD DNB should allow for a rough allele frequency approximation and resulted in 0.00065 and 0.00024 for *RHD* category VII and partial *RHD* DNB, respectively. These estimates are comparable to those of other reports [15, 16]. Other RhD categories

Table 1. Regular (upper block total n = 21) and variant (lower block, total n = 58) RhD phenotpyes investigateda

RhDCE, *RHD* investigated	n	RhD anti-gens/cell	RhCE haplotype	Allele FRQ[b]	Phene FRQ[c]	Reference antigen density	Reference frequency estimate	Medion, %		DiaMed, %		Bio-Rad, %	
								D1	D2	D1	DCE2	D1	D2
ccddee	1	D -		cde: 0.420	0.176	RhD -	this study	0	0	0	0	0	0
Ccddee	2	D -		.	0.013	RhD -	this study	0	0	0	100	0	0
CCddee	3	D -		Cde: 0.015	0.000	RhD -	this study	0	0	0	100	0	0
ccddEe	2	D -		.	0.006	RhD -	this study	0	0	0	88	0	0
ccddEE	2	D -		cdE: 0.007	0.000	RhD -	this study	0	0	0	100	0	0
ccDee	2	23,240		cDe: 0.017	0.014	19	this study	100	100	100	100	100	100
CcDee	2	13,283		.	0.361	19	this study	100	100	100	100	100	100
CCDee	3	22,778		CDe: 0.400	0.170	19	this study	94	94	100	100	100	96
ccDEe	2	19,710		.	0.122	19	this study	100	100	100	100	100	100
ccDEE	2	n.a.		cDE: 0.135	0.020	n.a.	this study	100	100	100	100	100	100
RHD DNB	3	5,908	CDe	0.00024	1:1,755	16	19, this study	72	72	100	100	75	88
RHD DFR	4	n.a.	CDe	n.a.	(>11)	n.a.	14	21	21	6	97	0	88
Rh33	1	n.a.	cDe	n.a.	(>11)	n.a.	21	33	33	75	88	25	0
RHD IV type 4	1	4,259	CDe	n.a.	(1)	20	14	67	67	100	100	81	88
RHD VI type 1	5	1,050	cDE	0.00076	1:1,494	19	7	0	0	0	88	0	0
RHD VI type 2	2	2,886	CDe	n.a.	(>11)	19	14	0	0	0	100	0	0
RHD VII	4	8,398	CDe	0.00064	1:4,680	19	15, this study	63	67	97	100	75	81
Weak *RHD* type 1	4	1,285	CDe	0.00217	1:521	19	7	33	29	41	100	34	47
Weak *RHD* type 2	5	489	cDE	0.00050	1:2,240	19	7	33	30	40	95	20	40
Weak *RHD* type 3	4	1,932	CDe	0.00328	1:345	19	7	58	58	81	100	56	69
Weak *RHD* type 4[d]	3	2,288	cDe	0.00020	1:5,601	19	7	67	67	92	92	79	75
Weak *RHD* types 4.2	1	1,650	cDe	n.a.	(>11)	19	14	50	50	75	75	63	75
Weak *RHD* type 5	3	296	cDE	0.00020	1:5,601	19	7	33	33	17	100	0	17
Weak *RHD* type 15	3	297	cDE	n.a.	(>11)	19	6, 8, 11	0	0	0	96	0	0
Weak *RHD* type 26	2	70	CDe	0.00005	1:20,988	17	17	33	33	0	100	0	0
RHD(M295I)	4	31	CDe	0.00027	1:4,198	13	17	0	0	0	100	13	13
RHD(IVS3+1G>A)	1	<22	Cde	0.00005	1:20,988	13	17	0	0	0	88	0	0
RHD-CE(2–9)-Dhybrid	8	RhD-	Cde	0.00131	1:862	RhD -	17	0	0	0	97	0	0

n.a. = Not available.

[a]Results as percentage of regular RhD expression (100%) of the serological typings.

[b]'Allele FRQ' describes haplotype and allele frequency applicable where appropriate.

[c]'Phene-FRQ' means phenotype frequency, e.g. a coinheritance of a specific variant *RHD* allele together with a *RHD* negative haplotype. '(>11)', or '(1)' indicate the number of independent worldwide observations according to Avent and Reid [14].

[d]Weak *RHD* type 4 includes type 4.0 and 4.1.

Table 2. Specific additional ABO genotypes (n = 14) investigateda

ABO genotypes investigated	n	Serological appearance	Phene FRQ[b]	A, %		
				Medion	DiaMed	Bio-Rad
O1/O2	8	O	>1:100	0	0	0
O1/A'3'	3	A weak	1:1,000	100	67	92
O1/A2	3	A weak	>1:100	100	100	100

[a]Results as percentage of regular A expression (100%) of the serological typings
[b]'Phene-FRQ' means phenotype frequency, e.g. a coinheritance of a specific variant *ABO* allele together with an *ABO* O-Allele. All three A'3' alleles had an A302 (currently listed as 'ABO*A3.02.1.1?' in the dbRBC of NCBI [14]) specific DNA sequence, with one additional substitution only (unpublished allele).

Table 3. Antibody clones included in the three methods validated.

Reagent	Clone(s) Anti-D$^{VI-(1)}$	Clone(s) Anti-D$^{VI-(2)}$
MDmulticard ABO-D-Rh subgroups-K for patients	LDM-1, TH-28	LDM-3, RUM-1
DiaClon ABO/Rh for patients	LHM 59/20 (LDM3) 175–2	MS-26, MS-201 (D clones in anti-CDE)
Scangel Monoclonal ABO/RH1/K	B9A4-B2A6A6A1A1	H2D5D2F5

and other partial RhDs as the ones listed above were only observed once in our database and not found to be representative of our Caucasian population.

With respect to weak RhD, types 1–5, 15 and 26 were enrolled for our test panel. Allele frequency estimates were deduced from earlier studies and were highest at 0.00328 for weak *RHD* type 3, which represents a phenotype frequency of 0.00291 or 1 carrier among 345 individuals in our local population [7]. Weak *RHD* type 15 had been observed on several occasions independently of the first publication and was therefore and because of its behavior as a partial RhD considered for inclusion into the test panel [6, 8]. However, no direct frequency estimates could be obtained for this allele. Although observed in one family only, weak *RHD* type 26 was included as marker, probably representing the lowest level of antigen RhD density still detectable without adsorption-elution technology [17].

With respect to RhDs DELs, the two most representative types found in the Caucasian population, *RHD*(M295I) and *RHD*(IVS3+1G>A), were considered for our test panel [17]. Composition of the final test panel, also including published antigen densities for RhD, is given in table 1.

Configuration of the Test Panel with Weak ABO A Phenotypes
For ABO, genotypes O1O2 (n = 8), O1A2 (n = 3) and O1A'3'(n = 3) were used, mainly to interpret weak serological reactivity for blood group A specificity. All three A'3' alleles had an A302 (currently listed as 'ABO*A3.02.1.1?' in the dbRBC of the National Center of Biotechnology Information (NCBI) [18]) specific DNA sequence, with one additional identical substitution only (unpublished allele). Expression strength of the above described variant of A302 is drastically reduced in comparison to A_1, or A_2. Composition of the final ABO-specific test panel is given in table 2.

Commercial Products for Serological Typing
For performance validation the following card reagents were used: MDmulticard® ABO-D Rhsubgroups-K for patients (Medion Diagnostics, Düdingen, Switzerland), ID-System® (DiaClon ABO/Rh for patients, DiaClon Rh-subgroups+K) (DiaMed, Ottobrunn, Germany) and Scangel®-Monoclonal Rh/K phenotypes (Bio-Rad, Vienna, Austria), Scangel®-Monoclonal ABO/RH1/K (Bio-Rad). All anti-D clones used in the described techniques do not recognize RhD category DVI. The detailed clone description of the anti-D clones is shown in table 3.

MDmulticard is a lateral flow device with a central application zone and two detection areas, one to each site of the application zone. The detection areas are impregnated with the respective antibodies, Further, both detection areas contain an autocontrol spot (ctl) and a process control spot (val). Briefly, 50 µl of anticoagulated whole blood are mixed with 200 µl of a diluent (Diluent F; Medion Diagnostics). 100 µl of the resulting suspension are then pipetted to the application zone. After 30 s, 300 µl of the above diluent are added to the application zone. Results can be read and recorded after 5 min. Positive results are displayed as stable bands, whereas negative results are monitored by the absence of the respective band [22].

ID-System and Scangel are gel techniques. For direct blood group typing in this technique, erythrocytes are centrifuged through gel matrices each containing the respective antibody. Six such reaction columns are included in one gel card. In positive reactions, hemagglutinated erythrocytes are entrapped on top of or dispersed throughout the gel matrix. In case of a negative result, the single erythrocytes sediment to the bottom of the gel matrix [23].

Serological and DNA Typing
After reading and signing informed consent, blood was donated for investigation from previously DNA-typed individuals. All individuals' samples were investigated with the above mentioned serological methods by two different technicians in a blinded way and retyped on DNA level with reagents for *ABO*, *RHD*, *RHD* weak, and *RHD* zygosity typing as provided by Innotrain (Kronberg, Germany). Reaction strength was assessed by visual inspection and scaled with 0 (no reactivity), ½ (almost invisible reactivity), 1, 2, 3, and 4 (strongest reactivity). The numerical data were then converted in percentage values, e.g. ½ corresponded to 12.5% and 4 to 100% (complete agglutination), and mean values of the two independent assessments by the two technicians were calculated (see tables 1 and 2).

Results

Test Panel Configuration

The final test panel consisted of 93 blood samples in total, divided in 21 regular RhDCE phenotypes, 58 variant RhDs

Proof

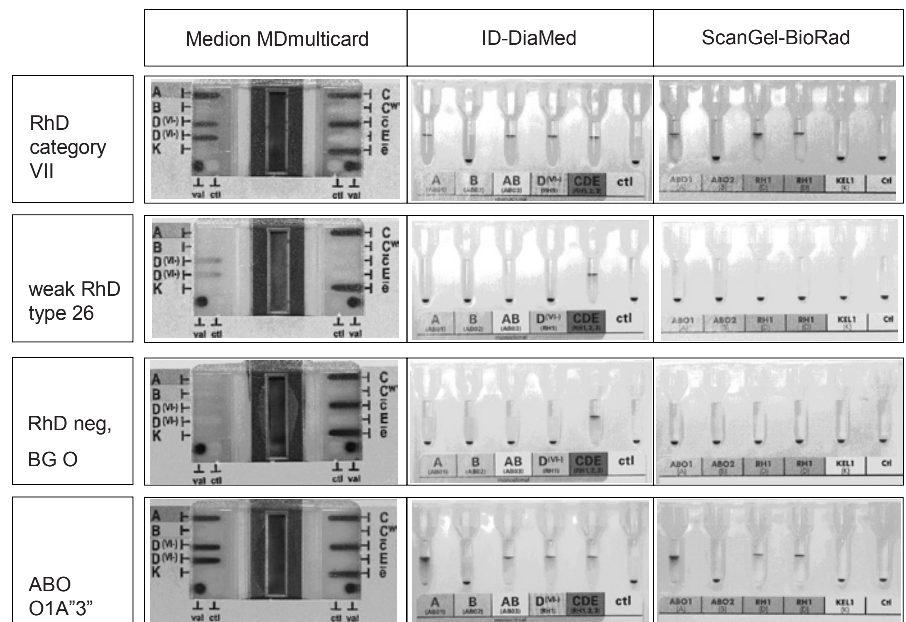

Fig. 1. Representative results for serological typings. Representative results for serological typings with focus on RhD are shown for the upper 2 samples: RhD category VII and weak RhD type 26. A RhD-negative and a 'regular' RhD-positive result can be seen in the lower 2 samples. Reactivity for weak RhD type 26 is weakly positive in the MDmulticard and negative in the other two methods. The sample with the A'3' allele had an A302 (currently listed as 'ABO*A3.02.1.1?' in the dbRBC of NCBI [14]) specific DNA sequence, with one additional substitution only (unpublished allele). Weakened reactivity of the A specificity of this sample is only seen by the ID-System and ScanGel methodology, whereas MDmulticard detects this sample as regular A.

– consisting of RhD categories and other partial RhDs, weak RhDs, RhD DELs and certain RhD-negatives – as well as 8 blood group O and 6 weak blood group A individuals (tables 1 and 2).

Range of Interpretation

The three methods included the following specificities:

- MDmulticard: A, B, 2 X D, K, C, Cw, c, E, e,
- ID-System: A, B, AB, D, CDE, C, c, E, e, K,
- Scangel: A, B, 2 X D, K, C, E, c, e, K.

This resulted in 930 single interpretation points for every method of the 93 samples investigated in total (tables 1 and 2). The discrepancy in the interpretation of the reaction strength of the same sample between the two technicians was never higher than 1 for any interpretation point in all three methods. Interpretation discrepancy totaled 29 for MDmulticard and 28 for ID-System or Scangel. Therefore, the discrepancy in the interpretation of the reaction strength between the two technicians was 29 (28) of 930, or 3.1% (3.0%) for all the three methods.

Typing for Regular RhD Positivity and Negativity, RhCcEe and K

Results for the typing of RhCcEe and Kell were identical, accurate, and as expected for all three serological methods compared. No variant pheno- or genotypes were included in the typing panel for these specific phenotypes. However, both RhD-specific reactions of MDmulticard and one of the two RhD-specific reactions of Scangel detected a slight weakening (94%) of the RhD positivity in 'regular' CCDee samples, whereas the CDE-specific reaction of ID-System found a slight weakened positivity (88%) in 'regular' ccddEe samples (table 1).

Typing for Variant RhD Expressivity

When focusing on specific RhD variant phenotypes, pronounced differences in reaction strengths were observed between the three methods. Exemplary results were: All RhD category VI samples were correctly typed as RhD-negative by all methods, as recommended for donors and pregnant women by certain national legislations. MDmulticard and Scangel defined RhD category VII and DNB samples as weakly RhD positive, whereas ID-System showed regular RhD positivity. Weak RhD type 5 was reliably detected as RhD-positive by MDmulticard, (33% in both reactions), but only very weakly by the ID-System (17%) and Scangel (0 and 17%). Astonishingly, weak RhD type 15 was recognized as RhD-negative by all three methods. Weak RhD type 26 was reliably recognized as RhD-positive by MDmulticard (33% in both reactions) only, whereas ID-System and Scangel interpreted it as RhD-negative in all typings. This is of specific interest since weak RhD type 26 has been shown to cause anti-D immunization when transfused to RhD-negative individuals and is thought to represent probably the lowest level of antigen RhD density still detectable without adsorption-elution technology [17]. On the other hand, DEL *RHD*(M295I), actually thought to be detectable by highly sensitive adsorption-elution techniques only, was reliably recognized as RhD-positive by Scangel (13% in both reactions). Detailed data of all results are given in table 1. Representative results for typings are given in figure 1.

Typing for Weak Blood Group A

All three methods reliably typed all regular blood group A, B, or AB phenotypes (n = 87), 100% accurately and specifically. With respect to A_2, of three *ABO**A2/O1 genotyped samples, the weak expression of A in these cases could not

be confirmed by any of the methods. With respect to A_3, of three *ABO**A'3'/O1 genoptyped samples, A was found regularly (100% (MDmulticard)), and weakly (67% (ID-System) and 92% (Scangel)) expressed by the three methods. Data are given in table 2, and representative typings are shown in figure 1.

Discussion and Conclusion

With respect to all common (regularly expressed) blood group phenotypes investigated, the results were correct and clear-cut positive or negative for all three methods compared (MDmulticard, ID-System, and Scangel).

With respect to ABO, only 3 distinct ABO genotypes were included in the presented test panel. Certainly, this is not 'representative' of any ABO polymorphism, independent of the kind of population investigated. The lack of ABO representativeness in our study is firstly reasoned by the lack of cumulative occurrence of certain variant ABO alleles included in our local database, i.e., carriers of certain variant ABO alleles were singular observations in practically all cases, beside the three different A phenotypes included in our study. Secondly, referring to our local database, variant ABO phenotypes were in general less frequently observed, than variant RhD phenotypes. In conclusion, A_2 (*ABO**A2O1 genotypes) was recognized as expressing A at regular strength by all three methods. However, recognizability of weak A samples is given as exemplified by a weak A encoded by an A302-like ABO allele investigated in the course of our study.

Looking at the serological typing for variant RhD phenotypes, only few results seem inaccurate. Among these, RhD weak D type 15 has not been detected by any of the evaluated serological methods. In order to minimize immunizations, this finding is of interest since no RhD-positive blood should be interpreted as RhD-negative. Moreover, RhD weak D type 15 has on average 297 RhD molecules on its cells, and recent data hint toward an inverse correlation between the number of transfused units and the probability of antibody formation, moving transfusion of minor amounts of RhD or weak RhD, as already shown for weak RhD type 26, into the focus of interest [17, 24]. On the other side, negative typing results for RhD weak D type 15 in pregnant women and recipients would lead to RhD prophylaxis or transfusion with RhD-negative blood. These treatments are professional in both cases and perfectly exemplify the two different standing points of donor and recipient typing in blood group diagnosis.

Other RhD phenotypes with unexpected negative typing results were *RHD* DFR: (6 and 0% with the ID-System) and one of the two RhD specificities of Scangel systems. We also found that among the 4 *RHD* DFR samples used for this evaluation differences had been observed in the serological results obtained with the three methods used, pointing to the fact that *RHD* DFR is defined by at least three alleles, presumably encoding different RhD epitope profiles [25]. At the time of investigation our DNA typing procedures were incapable of subtyping these three *RHD* DFR alleles. The differences in the serological results between the two RhD specificities of the Scangel system clearly indicated a difference in the respective antibody compositions, whereas the results of the two separate RhD specificities of the MDmulticard methodology were always identical.

With respect to weak RhD type 5, and type 26, only MDmulticard, recognized both reliably, whereas ID-System and Scangel both missed weak RhD type 26, with the same potential consequences as described above for weak RhD type 15. Additionally, weak RhD type 5 was only found to be agglutinated at a very low level (17% only) of the later two methods.

Focusing on those variant RhD phenotypes with doubtful serological typing results, their low RhD antigen density of on average 31 to 296 RhD molecules/cell becomes strikingly evident (no data available for *RHD* DFR). No other RhD phenotype with a comparably low antigen density had been included in this study and the next 'stronger' weak RhD type 2 with 489 molecules/cell was already typed reliably by all three methods. Therefore, a group of variant phenotypes consisting of e.g. weak RhD 5, 15 and 26 and others with comparably very low antigen densities may be perfect for the definition of the lowest detection limit for RhD seropositivity.

None of the above mentioned very-low antigen density RhDs – *RHD* DFR, weak *RHD* type 5, 15, and 26 – would have been included by statistical chance in a randomly chosen 3,000 sample panel as requested for performance validations. Our 79-sample panel for RhD serology however, included all regular phenotypes and all 'representative' rare variants and therefore actually corresponded to a much larger sample collection. The size of this sample collection can be calculated by the individual phenotype frequency of a single variant allele multiplied by their number of samples investigated during our evaluation. Exemplarily, investigating 4 samples of weak RhD type 5 with their local phenotype frequency of 1:5,601 individuals, multiplies to a final weak RhD type 5 'representative power' of 22,404 individuals. Representative powers of the 3 *RHD* DNBs, 5 *RHD* Category VI type 1, and weak *RHD* type 4.0 or 4.1 were 5,265, 5,976 and 16,803 in our population, respectively.

As described in Material and Methods, our 79-sample panel for RhD serology testing primarily resulted from sample availability among our donors. However, as expectable, but still by chance, the panel was considered as 'representative' of Caucasians when looking at allele frequency data, for example, it was inclusive of all *RHD* alleles having been observed more often than once or a few times only. As a guideline for the selection of 'representative' *RHD* alleles, frequency data as given in table 1 should be taken into account. In any case, we conclude that predefined multivariant test panels allow for more significant performance validations of new serological

test procedures than any reasonably sized panel based on statistical chances only. Of course, the same conclusion is applicable for other blood group specificities such as RhCE. In addition, we therefore recommend composition of the presented panel as an exemplary version for comparable performance validations.

Availability of such panels is a major task. The exclusive usage of fresh blood samples in the presented performance evaluation prevented from additional evaluation steps such as optimization of storage conditions in order to guarantee phenotype stability. With respect to ABO, Frame et al. [26] reported another interesting approach which could allow access to helpful reference material. Synthetic constructs incorporating A, B, acquired-B, and Le(a) blood group determinants were constructed and used to modify RBCs (KODE technology). Modified cells were then assessed by routine serologic methods using a range of commercially available monoclonal antibodies. Whether or not comparable methods will also allow the design of erythrocytes with respective protein moieties, e.g. RhD, is uncertain. However, as long as such artificial test erythrocytes are unavailable, defined blood donors are the only and best available resource for performance evaluations of serotyping methods.

Disclosure

The author declared no conflict of interest.

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