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Discrepancies between red cell phenotyping and genotyping in daily immunohematology laboratory practice

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ABSTRACT

False-positive and false-negative reactions exist for serological and molecular antigen typing methods. If the predicted phenotype is inconsistent with the patient`s known antibodies or serological phenotype, the discrepancy must be investigated. False-negative and false-positive results are clinically problematic in blood donors and patients. In this study, we investigated discrepant results between serology and molecular testing in patients and blood donors that occurred in daily molecular laboratory practice over a two year-period. SCD patients represented a large percentage of our cases of discrepancies but we also observed a high prevalence of discrepancies between phenotypes and genotypes in blood donors. The main reasons that led to discrepancies were recent transfusions and limitations of phenotyping. Discrepancies classified as false positive phenotype/true negative genotype and false negative phenotype/true positive genotype occurred mainly in patients with recent transfusions and individuals with RH variants while those classified as true negative phenotype/false positive genotype involved null phenotypes due to silent genes. Despite the limitations of molecular methods currently employed, we found more false-negative and false-positive phenotypes than genotypes demonstrating that genotyping is more efficient to define the blood types, especially in transfusion dependent patients.

1. Introduction

Hemagglutination has been used to determine red blood cell (RBC) types and has been considered the gold standard for over a century [1]. However, technical and clinical limitations of serologic immunohematology have led many laboratories to the use of molecular assays to predict red cell phenotype [2]. The characterization of the genes encoding the 36 blood group systems recognized by the Working Party on Red Cell Immunogenetics and Blood Group Terminology of the International Society of Blood Transfusion (ISBT) [3] and the knowledge of the molecular events that give rise to blood group antigens and phenotypes [4] have made possible the application and implementation of molecular testing into blood centers, reference laboratories and transfusion services [2,5].

Low-, medium- and high-throughput techniques have been developed for blood group genotyping and in the last decade we have seen a great expansion and evolution of the technologies available [6–12]. Thus, molecular testing is rapidly advancing and offers tremendous help as a powerful tool with potential advantages in the identification of rare RBC donors and finding antigen matches for chronically transfused patients [7,13–17]. However, it should be noted that, regardless of the test protocols used, genotyping predicts a blood type but does not determine the phenotype the way serologic tests do. In some instances, the genotype will not correlate with the phenotype because the simple presence of a gene does not mean that the gene will be expressed as an antigen on the RBC membrane. A large number of genetic events may silence or weaken the expression of antigens encoded by an allele [4]. There are several examples of misleading results of molecular typing in the literature, but there is a consensus that molecular typing test is an invaluable supplement to traditional serological method and these tests are likely to become essential, rather than optional, for blood donor and patient testing [15,16]. Thus, the profile of a gene needs to be completely elucidated, and appropriate assays need to be performed to look for genetic changes that may alter the predicted phenotype.

False-positive and false-negative reactions exist for serological and molecular antigen typing methods. If the predicted phenotype is inconsistent with the patient`s known antibodies or serological phenotype, the discrepancy must be investigated. False-negative results are clinically problematic in blood donors as they may induce alloimmunization in patients and false-positive results are relevant for patients as they may produce antibodies to antigens that they do not actually have. The resolution of serological and molecular discrepancies, besides providing a correct blood unit selection for recipients, may also contribute to the identification of new alleles and blood group antigens.

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Besides of equipment and human failures, many reasons may explain discrepancies between phenotyping and genotyping results. The aim of this study was to assess the discrepancies between phenotyping and genotyping results that occurred in our daily molecular routine in a two-year period, to identify the causes, the way they were solved and to classify them according to the type of discrepancy.

2. Materials and methods

2.1. Population studied

In a two-year period, from 2015 to 2017, samples referred to our molecular laboratory with discrepant results between phenotyping and genotyping were studied under an institutional review board-approval protocol. In this period, a total of 734 genotyping assays were requested to our molecular laboratory but due to the lack of information 282 cases were excluded and therefore we analyzed 452 cases. All patient's informations' including diagnoses and alloimmunization history were reviewed.

2.2. Serologic and molecular analyses

RBC antigen phenotypes of each donor and patient involved in this study were obtained from the medical records and transfusion service computerized database. Antibodies were classified as auto or alloantibodies based on the results of both, serological and molecular testing. Depending on the type of discrepancy, donors and patients were invited to perform new serological tests. Genomic DNA was extracted from buffy coat of peripheral blood from patients and donors with QIAamp DNA Blood Mini Kit (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's instructions. RBC genotyping was performed using conventional PCR assays and wHEA, wRHD and wRHCE BeadChip arrays (BioArray Solutions, Immucor, Norcross, GA, USA) in accordance with the manufacturer's instructions. DNA sequence analysis was performed on PCR products amplified from gDNA in all samples that were not characterized by conventional PCR assays or BeadChip arrays.

In order to determine *RH* allelic combinations on samples identified with *RHCE* variants, we performed Rh-cDNA cloning and sequencing. RNA was isolated from reticulocytes with TriZol (Invitrogen, Carlsbad, CA). Reverse transcription (RT) was carried out with Superscript First Strand Synthesis (Invitrogen, Carlsbad, CA) using gene-specific primers. PCR products, amplified from cDNA, were purified with ExoSAP-IT (USB, Cleveland, OH), cloned into a TA vector (Invitrogen, Carlsbad, CA) and sequenced using primers, as previously reported [17].

For specific detection of the *RHD* gene deletion, we used PCR-RFLP amplification of the downstream and hybrid Rhesus box as well as digestion of the PCR products with the restriction enzyme *Pst* I, as previously reported [18]. We also used a quantitative PCR approach [19], complemented by the specific detection of *RHD*Ψ [20].

2.3. Classification of discrepancies

The reported phenotype results were initially compared to genotyping results and ranked among patients and donors. Patients were further classified according to their pathologies. According to the type of discrepancies, we tried to classify our results in four possible combinations: false positive phenotype/true negative genotype; false negative phenotype/true positive genotype; true negative phenotype/false positive genotype; true positive phenotype/false negative genotype.

3. Results

From 452 cases studied referred to our laboratory, 325 (71.9%) had discrepancies between phenotyping and genotyping.

Table 1 shows the distribution of the discrepancies according to the clinical condition of each individual (donors and patients). We classified the patients according to their diagnoses and transfusion need as Sickle Cell Disease (SCD), Thalassemia, Auto-Immune Hemolytic Anemia (AIHA) and other pathologies. There were patients with more than one type of discrepancies and therefore from 325 cases analyzed, we had a total of 386 types of discrepancies between serology and molecular testing results. Among the polytransfused patients, SCD patients were those with a greater number of discrepancies (22.02%), followed by thalassemia (5.44%) and patients with AIHA (4.92%). Other pathologies represented 53.89% of the discrepancies and included patients with neoplasia, fractures, among others. Patients with an unidentified diagnosis were also classified as "other pathologies". It is noteworthy the number of donors that presented discrepancies between phenotyping and genotyping (13.73%). The main reasons of discrepancies in the patients were recent transfusions, positive direct antiglobulin test (DAT), silent alleles and RH variants. In blood donors the main cause of discrepancies was the presence of RH variants. According to our results, the discrepancies between phenotype and genotype results, were classified in 3 combinations: false positive phenotype/true negative genotype; false negative phenotype/true positive genotype; true negative phenotype/false positive genotype. We did not obtain any discrepant results that could be included in the fourth possible combination: true positive phenotype/false negative genotype.

Table 1
Distribution of the discrepancies between phenotype and genotype results according to the clinical condition of the individuals.

Individuals	Discrepancies	
	n	%
SCD patients	85	22.02
Thalassemia patients	21	5.44
Patients with AIHA	19	4.92
Other patients	208	53.89
Donors	53	13.73
Total	386	100

3.1. False positive phenotype/true negative genotype

The number of discrepancies classified in this combination according to the clinical condition of the individuals are shown in Table 2. Sixty (15.5%) types of discrepancies between serology and molecular testing results were classified having as main reasons: positive DAT, patients recently transfused and the presence of Rh variants. The most common types of discrepancies found are displayed in Table 3.

Table 2
Number of discrepancies between phenotype and genotype results classified as false positive phenotype/true negative genotype, false negative phenotype/true positive genotype and true negative phenotype/false positive according to the clinical condition of the individuals.

Individuals	False positive phenotype/true negative genotype		False negative phenotype/true positive genotype		True negative phenotype/false positive genotype	
	n	%	n	%	n	%
SCD patients	10	2.59	32	8.29	43	11.13
Thalassemia patients	9	2.33	14	3.62	3	0.77
Patients with AIHA	3	0.77	5	1.29	5	1.29
Other patients	34	8.8	40	36.26	34	8.8
Donors	4	1.03	140	12.17	3	0.777
Total	60	15.52	238	61.63	88	22.76

Table 3
Examples of the most common types of discrepancies found between phenotype and genotype results classified as false positive phenotype/true negative genotype.

Causes of discrepancies	Discrepant results	
	Phenotype	Genotype
Positive DAT	C + c + E + e +	*RHCE*Ce/RHCE*ce*
	Jk(a + b +)	*JK*B/JK*B*
Antigen-negative patient recently transfused with antigen-positive RBCs	C + c + E + e +	*RHCE*Ce/RHCE*ce*
	C–c + E + e +	*RHCE*cE/RHCE*cE*
	Fy(a + b +)	*FY*B/FY*B*
	Jk(a + b +)	*JK*A/JK*A*
Rh variants	D +	*RHD–, RHCE*ceHAR*
	C +	*(C)ce^{S} (RHD-CE(4-7)-D)*

3.2. False negative phenotype/true positive genotype

The number of discrepancies classified in this combination according to the clinical condition of the individuals are shown in Table 2. Two hundred and thirty-eight (61.6%) types of discrepancies were included in this classification. Here, the main reason of discrepancies between phenotype and genotyping testing results were the presence of RH variants (51%), especially *RHD* variants, followed by recent transfusions, and the weak expression of Fy^{b} (Fy^{x}). The most common types of discrepancies found are displayed in Table 4.

3.3. True negative phenotype/false positive genotype

In this combination we found 88 (22.7%) discrepancies between phenotype and genotype results. The types of discrepancies classified as true negative phenotype - false positive genotype have as main reasons the silent alleles, especially the -67C mutation in the GATA box promoter sequence of FY gene, representing 89,8% of the cases. Other silent alleles included mutations in the coding sequence of *RH, KEL, DO*, *LU*, and *JK* genes identified by sequencing (Table 5). The numbers of discrepancies in this combination according to the clinical condition of the individuals are displayed in Table 2.

3.4. Discrepancies between phenotype and genotype results and blood group systems

Table 6 shows the classification of discrepancies by blood group system. We observed that the discrepancies involving the Rh blood group system were the most frequent in this cohort, followed by FY blood group system.

Table 4
Examples of the most common types of discrepancies found between phenotype and genotype results classified as false negative phenotype/true positive genotype.

Causes of discrepancies	Discrepant results	
	Phenotype	Genotype
Weak antigen expression	D–	*RHD*weak D type 2*
	D–	*RHD*weak D type 38*
	D–	*RHD*DEL1*
	Fy(b–)	*FY*02W.01*
Partial antigen	D–	*RHD*DAR*
	D–	*RHD*DVI*
Antigen-positive patient recently transfused with antigen-negative RBCs	C–c + E–e +	*RHCE*Ce/RHCE*ce*
	C–c + E–e +	*RHCE*cE/RHCE*ce*
	Fy(a–b +)	*FY*A/FY*B*
	Jk(a + b–)	*JK*A/JK*B*
Poor quality of antisera	U–	*GYPB*P2* (U + ^{var})

Table 5
Discrepancies classified as true negative phenotype/false positive genotype involving null phenotypes that could impact in the transfusion decision.

Blood group system	Phenotype	Genotype	N
RH	D–, C–, E–, c–, e–	*RHD–, RHCE*C/c, RHCE*e/e*	1
RH	D + , C–, E–, c–, e–	*RHD + , RHCE*c/c, RHCE*e/e*	2
KEL	K–k–, Kp(a–b–) Js (a–b–)	*KEL*02, KEL*02.04, KEL*02.07*	2
JK	Jk(a–)	*JK*A/JK*B*	1
LU	Lu(a–b–)	*LU*B/LU*B*	2
DO	Do(a–b–), Hy–, Jo(a–)	*DO*A/DO*A, HY + , JO*A*	1
Total			9

4. Discussion

Accurate RBC typing of patients and blood donors is essential to prevent alloimmunization and hemolytic transfusion reactions. Molecular typing has been implemented in Immunohematology laboratories in order to overcome the limitations of hemagglutination, improving transfusion safety. However, DNA analysis has also some limitations that can lead to false-positive or false-negative phenotype predictions as the methods currently used are only capable of detecting those alleles included in the assay design. But despite the limitations, genomics is considered an essential tool in the Immunohematology laboratories. This study evaluated the number and types of discrepancies found in a daily molecular laboratory practice. Our study analyzed all discrepant results between serology and molecular testing in patients with different diagnoses and blood donors over a two year-period.

When analyzing individually the discrepancies found, we observed that SCD patients represent a large percentage of our cases of discrepancies. In addition to their transfusion need, the genetic inheritance that African descendants carry and that influence the expression of RBC antigens can be considered responsible for the high rate of discrepancies in these patients. Our results are in agreement with other studies [21–25] showing that these patients are more susceptible to alloimmunization and present more discrepancies between phenotypes and genotypes than other types of patients.

An interesting finding in our study was the high prevalence of discrepancies between phenotypes and genotypes in blood donors (13.73%). This result is consistent with the results obtained by Chang et al. [26] who evaluated 133 donors comparing their serological and molecular tests and found 17 (12.8%) subjects with discrepancy between phenotyping and genotyping with a total of 19 antigens involved, especially within the RH system. Many of the discrepancies we found were consequences of altered expression of Rh antigens leading to false-negative phenotypes and are probably a result of the heterogeneous ethnic background of our donor population composed of 75% European, 18% African and 7% Amerindian ancestry.

When we analyzed the reasons that led to discrepancies between phenotype and genotype results, we observed that the main reasons to explain the discrepancies were: recent transfusions, difficulties in differentiation of auto and alloantibodies, limitations of phenotyping due to poor or unavailable antisera, positive antiglobulin test (DAT), weak and partial phenotypes.

Discrepancies classified as false positive phenotype/true negative genotype and false negative phenotype/true positive genotype occurred mainly in patients with recent transfusions demonstrating once again that phenotyping is compromised in these patients. “RhD and RhCE” variants were also predominant in this type of classification, which demonstrates that there is an important serological limitation in the identification of these variants, as previously reported [5,7,22,25].

Discrepancies classified as true negative phenotype/false positive genotype involved null phenotypes due to silent genes. From 88

Table 6
Classification of discrepancies between serology and molecular testing results by blood group system according to the clinical condition of the individuals.

Individuals	Blood group systems							Total
	RH n (%)	KEL n (%)	FY n (%)	JK n (%)	MNS n (%)	DI n (%)	DO n (%)	n (%)
SCD	30 (7.8)	3 (0.8)	46 (11.9)	1 (0.26)	3 (1)	1 (0.26)	1 (0.26)	85 (22.02)
Thalassemia	13 (3.4)	0	7 (1.8)	1 (0.26)	0	0	0	21 (5.4)
AIHA	5 (1.3)	3 (0.8)	6 (1.5)	0	4 (1)	1 (0.26)	0	19 (4.9)
Others	138 (35.7)	12 (3.1)	41 (10.6)	4 (1)	12(3.1)	1 (0.26)	0	208 (53.9)
Donors	49 (12.7)	0	1 (0.26)	3 (0.8)	0	0	0	53 (13.7)
Total	235 (60.9)	18 (4.6)	101 (26.2)	9 (2.3)	19 (4.9)	3 (0.77)	1 (0.26)	386 (100)

individuals with this type of discrepancies 79 were phenotyped as Fy (a–b–) with a homozygous -67T > C mutation in the GATA box promoter sequence. We included this *FY* silent gene in this classification because the discrepancy is observed when comparing the phenotypes and genotypes results, even though the HEA BeadChip Kit used to genotype our samples can identify the GATA mutation and predict the Fy(a–b–) phenotype. Thus, considering this fact, we would have in this group only 9 true false-positive genotypes that could impact in the transfusion decision (Table 5).

Regarding blood group systems, RH and FY were the most prevalent systems involved with discrepancies between phenotype and genotype results. This finding may reflect the population studied with heterogeneous ethnic background and a high degree of admixture between Europeans and Africans as the Brazilian population.

When we evaluated the discrepancies between serology and molecular typing we observed that despite the limitations of molecular methods currently employed, we found more false-negative and false-positive phenotypes than genotypes with no false negative genotype demonstrating that genotyping is the most efficient method for determining blood group types, especially in transfused dependent patients. However, we need to be aware that an error in determining a blood group either by phenotype or genotype can have serious consequences for the patient receiving transfusions [27]. Thus, the integration of serological and molecular tests in the Immunohematology routine as well as the evaluation, resolution and classification of the discrepancies found will help in the correct interpretation of the results found and, consequently, in the increase of transfusion safety.

5. Conclusion

Serological identification of RBC antigens is important in the search of compatible blood in transfusion medicine. Molecular tests complement the analysis and favors the identification of the correct phenotype or the presence of variants that must be considered for transfusion safety. It is, however, essential to take into account that a genotype is only the deduction of a phenotype, especially when related to the presence of silent genes and absence of DNA amplification due to unexpected mutations. Discrepancies between serology and molecular testing results are common and more associated with false negative and false positive phenotype results. The full replacement of blood group phenotyping by molecular testing is still a matter of debate, but with the advance and implementation of next generation sequencing tests in the clinical setting, this could be a reality in a near future.

Authorship contributions

Sheila FP Menegati, carried out the molecular genetic studies, analyzed data and wrote the manuscript, Tamires Delfino dos Santos and Mayra D Macedo carried out molecular analyses. Lilian Castilho designed and coordinated the study, analyzed data and reviewed the manuscript. All authors read and approved the final manuscript.

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