ARTICLE IN PRESS

Transfusion and Apheresis Science xxx (xxxx) xxxx

Contents lists available at ScienceDirect

Transfusion and Apheresis Science

journal homepage: www.elsevier.com/locate/transci

Anti-A and anti-A,B monoclonal antisera with high titers favor the detection of A weak phenotypes

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ARTICLE INFO

Keywords:
ABO subgroups
Red blood cell phenotyping
Blood group genotyping
Antigen-antibody reactions

ABSTRACT

Objectives: This study aimed to evaluate the reactivity and the titers of commercial anti-A and anti-A,B antisera in the detection of A weak antigen expression in human red blood cells.
Background: Commercial monoclonal antisera for ABO phenotyping are useful reagents allowing the identification of the four main ABO phenotypes (A, B, AB, and O). However, the reactivity of these commercial reagents can not be evident when the A or B antigens are weakly expressed, and these antisera have low titers.
Methods/Materials: Six samples from blood donors and five samples from patients with ABO forward and reverse discrepant phenotyping were evaluated. The ABO phenotyping was carried out with different commercial monoclonal anti-A and anti-A,B antisera under different temperatures, using test tubes and gel column agglutination.
Results: Monoclonal anti-A antisera with titers less than 256 and anti-A,B with titers less than 128 failed to detect the weak expression of A antigen in 73% and 67% of the A weak phenotypes, respectively. Titres equal to or higher than 2048 (anti-A) and 1024 (anti-A,B) showed better reactivity, independent of the cell clone.
Conclusion: Our data indicate that anti-A and anti-A,B antisera with high titers give better reactivity with red blood cells carrying A weak antigen expression.

1. Introduction

The serological identification of the four main ABO phenotypes requires the forward and reverse phenotyping tests. The results of both tests must be complementary to allow the correct phenotyping [1]. However, under some circumstances, discrepancies, which are defined as inconsistent results between forward and reverse phenotyping, can occur due to weak antigen expression on the red blood cells, faint expression of regular anti-A, and or anti-B antibodies, among others [1–4]. These discrepancies can be transitory as results of some diseases (cancer, leukemia, for instance) or resulting from polymorphisms in the exons of the ABO gene coding the functional region of the glycosyltransferases [3–7].

The ABO gene located at chromosome 9 (q34.2) contains various single nucleotide polymorphisms, which reduces the affinity of the encoded glycosyltransferases for the H antigen [6,7]. Additionally, the availability of this substrate within the Golgi apparatus affects the level of A and B antigens expressed in the red blood cell membrane creating A or B weak phenotypes, which can present discrepancies between forward and reverse phenotyping [8–11].

Most of the discrepancies observed in the ABO forward and reverse phenotyping can be solved with an extensive serological approach to confirm the presence or absence of the A or B antigens [1,12]. However, the resolution of some discrepancies is not so simple. Sometimes it is necessary to associate different serological methods with distinct cell clones combined with molecular analysis [13,14]. Even thus, some cases present complexities that are not always resolved by serological and molecular analysis.

Some studies analyzed the reactivity of ABO commercial antisera using red blood cells with weak A antigen expression. One of them, based on the data of a reference laboratory, reported variability in the reactivity of monoclonal anti-A and anti-A,B commercial antisera [15]. The other one also observed differences in the reactivity of monoclonal antibodies tested in forwarding phenotyping (anti-A non-reactive, and anti-A,B weakly reactive) [13]. These studies demonstrate that ABO commercial monoclonal antisera might fail in cases where the A and or

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https://doi.org/10.1016/j.transci.2020.102865
Received 29 April 2020; Received in revised form 4 June 2020; Accepted 18 June 2020

B antigens are faintly expressed.

The reports above mentioned rise up an essential concern about the inability of commercial antisera in detecting A weak antigen expression in human red blood cells. However, these reports did not evaluate the titers of commercial antisera used to ABO phenotyping. This study evaluates the reactivity and the titers of commercial anti-A, anti-A,B antisera commercialized by different suppliers in the detection of A weak antigen expression in human red blood cells.

2. Material and methods

2.1. Ethical aspects of the research

This study was approved by the Ethics and Research Committee from Faculdade de Medicina de São José do Rio Preto (case 784.164). All participants were informed about the objectives of the study and signed the informed consent form.

2.2. Casuistic

Six samples from blood donors (BD) and five samples from patients (PT) with ABO forward and reverse phenotyping discrepancies or discordant results concerning previous annotations, were sent to our laboratory for additional investigations. All the BD were able to donate, and the PT had a history of a medical prescription for transfusion due to elective surgery or low hemoglobin levels. No information about the physiopathological evidence that could affect the expression of red blood cell ABO antigens or serum antibodies from all BD and all PT were informed to our laboratory by senders. Table 1 shows the data of the BD and PT as informed by sample senders.

2.3. Adopted criteria to recognize discrepancies in the ABO phenotyping

We adopted the following criteria to identify an ABO discrepancy: inconsistent results between the forward and the reverse ABO phenotyping; unexpected reactions that might hide a potential discrepancy; divergence between previous and current results reported by the sample senders (Table 1). The variant ABO phenotypes were assigned in general terms as A_{weak} in cases where the subgroup status could not be determined.

2.4. Standard phenotyping

Forward and reverse ABO phenotyping were carried out using standard test tubes with commercial antisera anti-A (cell lines: 9113D10, Birma-1, and 11H5), anti-B (cell lines: 9621A8, LB-2, and ES-4), and Anti-A,B (cell lines: 9113D10 + 152D12, ES-15 + Birma-1 + LB-2, and ES-15 + Birma-1 + ES-4) from five different manufacturers. Additionally, ABO phenotyping was performed: 1 - using commercial gel column agglutination (CAT) [ID-card ABO/D + Reverse Grouping (CAT 1)], containing monoclonal anti-A (cell line A5) and anti-B (Cell lines: G1/2) antibodies; 2 - ID-Card DiaClon ABO/Rh for newborns (CAT 2) containing the monoclonal anti-A [Cell lines: A: LM297/628 (LA-2)], and anti-B [Cell line: LM306/686 (LB-2)], and anti-A,B [Cell line: ES131 (ES-15), Birma-1, ES-4] antibodies. Commercial A_1 and B red blood cells (DiaMed - Latin America, Brazil) were used in both test tubes and CAT for reverse phenotyping. The red blood cells from blood donors and patients were tested with anti-A1 (*Dolichos biflorus*) and anti-H (*Ulex europaeus*) lectins in tubes as well as in CAT. All manufacturers' instructions were strictly followed.

2.5. Modified phenotyping

We performed a modified forward and reverse ABO phenotyping using different temperatures and incubation times as well as red blood cells treated with enzymes (bromelain) to potentialize the agglutination reactions [1]. Adsorption and elution tests were also performed. All these tests were performed using ID-Card NaCl (Neutral gel: NG) and ID-Diluent 1 Bromelain (DiaMed - Latin America, Brazil) with the same commercial antisera and red blood cells.

2.6. Titration of anti-sera anti-A and anti-A,B

All commercial antisera anti-A and anti-A,B used were previously titrated using A_1, A_2, and A_2B red blood cells, according to American Association Blood Bank's protocol [1], and with the certificates of analysis provided by the commercial suppliers, and the recommendations from Brazilian Health Ministry legislation for transfusional medicine [16]. We used antisera from the different commercial suppliers in Brazil identified as M1, M2, M3, M4.1, M4.2, and M5. Some of them belong to the same cell clone and two (M4.1 and M4.2), from the same supplier.

2.7. Extraction of genomic DNA

Genomic DNA from white blood cells was extracted using the QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany). All manufacturers' instructions were strictly followed.

2.8. Molecular analysis

We used a protocol to analyze the single nucleotide polymorphism 261delG of the exon 6 by PCR-RFLP as well as amplified fragments from exon 2 to exon 7 of the ABO gene for sequencing analysis [17]. The amplified fragments from these exons were sequenced using commercial kits BigDye Terminator and ABI PRISM 3500 Genetic Analyzer

Table 1
Data from blood donors (BD) and patients (PT) as informed by sample senders.

Cases	ABO Grouping			Previous observations
	Red Cells	Serum	Previous record	
C1-PT	O	A	O	A antigen not investigated in previous sample.
C2-PT	AB	B	NA	Negative agglutination with two different anti-A.
C10-BD	O	A	NA	A antigen investigated but not detected.
C14-BD	B	B	AB	Phenotype divergent from the previous donation.
C18-BD	AB	AB	B	Phenotype divergent from the previous donation.
C21-BD	AB	B	NA	Presence of an irregular anti-A1.
C25-BD	O	A	O	Divergent, positive and negative eluate results
C26-PT	O	A	NA	Positive with anti-A,B monoclonal antibody
C27-PT	O	A	NA	Positive with anti-A,B monoclonal antibody
C28-PT	O	A	NA	Divergent, eluate not investigated
C31-BD	AB	B	B	Phenotype divergent from the previous donation.

NA: data not available.

(Applied Biosystems, Foster City, CA, EUA). The Sequence Scanner Software 2 (Applied Biosystems Software) and the BioEdit sequence alignment editor were used [18]. The ABO sequences obtained were aligned with the NCBI Reference Sequence for *ABO*A1.01* (NG_006669.1) using the software BioEdit [18].

2.9. Score agglutination

The agglutination score followed the proposition by Marsh [19], revised by the American Association of Blood Banks [20].

2.10. Molecular adopted nomenclature

We adopted the terminology for the ABO gene and alleles, according to the International Society of Blood Transfusion (ISBT) (www.isbtweb.org) [21].

3. Results

3.1. Serological data

The results of the serological analysis are showed in Table 2. All the samples evaluated (six A, five AB) presented weak expression of A antigen according to the agglutination score adopted. An irregular anti-A1 antibody was detected in four samples (C2-PT, C14-BD, C21-BD, C31-BD).

The C10-BD sample showed reactivity only in the eluate test. The red blood cells from the other samples showed distinct reactivity in the forward phenotyping when tested with antisera anti-A in CAT, independent of the treatment or non-treatment with the enzymes.

The samples C2-PT, C14-BD, C21-BD, and C31-BD showed reactivity in the reverse phenotyping in test tubes with A red blood cells. On the other hand, the remaining samples (except C18-BD) presented distinct reactivity with A red blood cells in reverse phenotyping, in the modified methods. All samples did not react with anti-A1 lectin but reacted with anti-H lectin. All eluates presented reactivity with A red blood cells.

Table 3 shows the results of the serological evaluation of the samples with anti-A and anti-A,B antisera, with different titration. We observed that the anti-A and anti-A,B antisera from the same cell clone, but commercialized by different manufacturers and presenting different titrations, showed distinct reactivity with samples carrying the weak expression of the A antigen in test tubes and CAT at 4 °C and 22 °C. Except for C10-BD sample, all other samples presented better reactivity in modified methods from antisera with high titrations.

The antisera anti-A from cell clone 9113D10, commercialized by different suppliers, presented the same reactivity with all samples when the titrations were equivalent, except for the C10-BD sample. The behavior of the antisera anti-A from cell clone BIRMA-1 was equivalent to the antisera anti-A from cell clone 9113D10 under the same conditions. The anti-A antisera from cell clone 11H5 (titer 512) presented similar reactivity to the anti-A antisera from cell clones 9113D10 and BIRMA-1 (titer 1024) (Table 3).

3.2. ABO molecular analysis by PCR-RFLP and sequencing

The common deletion 261delG at the exon 6 was identified by PCR-RFLP only in the samples C1-PT, C25-BD, C26-PT, C27-BD, C28-BD, and C10-BD. The sequencing analysis confirmed the heterozygote genotype, with an A allele and one deletional O allele. An insertion c.804 in. G at exon 7 was found at the sample C10-BD, which is compatible with the *ABO*AEL.01* allele. The sequencing of the exons 2 to 7 from sample C25-BD revelead the presence of the mutations c.297A > G, c.646T > A, c.681G > A, c.771C > T, and c.829G > A which allowed us to identify the allele *ABO*AW31.01*. The sequencing of the sample C31-BD showed the mutations c.46G > A, c.106G > T, c.188G > A, c.220C > T, c.467C > T and c.1061delC which allowed the identification of the allele *ABO*AW.09*.

The samples C1-PT, C2-PT, C-14-BD, C18-BD, C21-BD, C26-PT, C27-PT, C28-PT were sequenced in the exons 6 and 7 and the only the mutations c.467C > T and c.1061delC were identified in all of them. The samples carrying the AB phenotype presented the single nucleotide polymorphisms (c.297A > G, c.526C > G; c.657C > T, c.703G > A, c.796C > A, c.803G > C, c.930G > A) that allowed to confirm the presence of the B allele in these cases.

4. Discussion

This study evaluated eleven blood samples from BD and PT, which presented discrepancies in the forward and reverse phenotyping, or discordant results concerning previous annotations in the medical records informed by the sample senders. To resolve these cases, we explored a serological approach determining the titration of the commercial antisera in combination with molecular analysis based on PCR-RFLP and sequencing. These strategies allowed us to demonstrate the presence of A antigen through the reactivity of eluates from all samples as well as infer the presence of a functional A weak allele in the genotypes through the molecular approach. By the author's knowledge, this is the first study evaluating the commercial antisera titration applied to detect the weak expression of A antigen.

Our data showed variability in the weak reactivity of red blood cell samples evaluated, which seems to be dependent on the titration of anti-A and anti-A,B commercial antisera, but not necessarily from the cell clone from which they come. Previous observations support the view that the serological performance of the antisera correlates their

Table 2
Serological and molecular data from ten cases presenting the weak expression of A antigen.

Cases	Antisera (FP)			Red blood cell (RP)		Lectins		Eluates	Phenotypes	Genotypes
	A	B	AB	RA	RB	A_1	H	A_1		
C1-PT	0	0	0	0	4	0	4	4	A_{weak}	*A/O*
C25-BD	0	0	0	0	4	0	4	4	A_{weak}	*A/O*
C26-PT	0	0	W	0	4	0	4	3	A_{weak}	*A/O*
C27-PT	0	0	1	0	4	0	4	4	A_{weak}	*A/O*
C28-PT	0	0	0	0	4	0	4	3	A_{weak}	*A/O*
C10-BD	0	0	0	0	4	0	4	2	A_{el}	*A/O*
C2-PT	w	4	4	0	0	0	2	4	$A_{weak}B$	*A/B*
C14-BD	0	4	4	2	0	0	2	4	$A_{weak}B$	*A/B*
C18-BD	1	4	4	0	0	0	2	4	$A_{weak}B$	*A/B*
C21-BD	1	4	4	1	0	0	2	4	$A_{weak}B$	*A/B*
C31-BD	1	4	4	2	0	0	2	4	$A_{weak}B$	*A/B*

FP: Forward Phenotyping; RP: Reverse Phenotyping; Results of the phenotyping ABO standard method using CAT 1 and CAT 2; Anti-A_1 was reactive in C2-PT and showed stronger results in C14-BD, C21-BD, and C31-BD in the tube test (results not shown).

Table 3
Evaluation of the reactivity of the antiserum with predetermined titers.

Antisera	Cell clones	Suppliers[a]	Titration[b] A_2	Cases										
				C1-PT	C25-BD[c]	C26-PT	C27-PT	C28-PT	C10-BD[d]	C2-PT	C14-BD	C18-BD	C21-BD	C31-BD[e]
Anti-A	9113D10	M1	256	0	0	0	W	0	0	1	0	0	W	0
		M3	1024	1	0	0	1	W	0	3	1	MF	MF	W
		M2	2048	2	1	1	2	1	0	3	1	2	3	1
	BIRMA-1	M4.1	128	0	0	0	0	0	0	0	0	0	0	0
		M4.2	1024	–	W	0	1	W	0	–	W	MF	MF	W
	11H5	M5	512	–	0	0	1	0	0	–	W	MF	MF	0
Anti-A,B	9113D10, 152D12	M1	128	0	0	0	2	MF	0	–	–	–	–	–
		M2	256	2	0	0	3	MF	0	–	–	–	–	–
		M3	1024	2	3	1	4	4	0	–	–	–	–	–
	ES-15, BIRMA-1, LB-2	M4.1	64	0	0	0	W	0	0	–	–	–	–	–
		M4.2	2048	–	4	2	4	4	0	–	–	–	–	–
	ES-15, BIRMA-1, ES-4	M5	512	–	0	0	4	3	0	–	–	–	–	–

MF: Mixed Field; Results of the phenotyping ABO using ID-Card NaCl at 22 °C.
[a] Different commercial suppliers in Brazil.
[b] The titer was previously determined with A_2 red blood cells.
[c] *ABO*AW31.01*.
[d] *ABO*AEL.01.01*.
[e] *ABO*AW09.01*.

antibody concentrations [22]. Besides, the ability of ABO blood grouping reagents to detect low levels of antigens in ABO subgroups can be influenced by the concentration or titer of the anti-A and anti-B antibodies [23,24].

We observed that the anti-A from cell clone 9113D10 reacted only with three samples at the titration 256 but reacted with 9 samples at the titration 2048. Similar reactivity was observed with anti-A from cell clone BIRMA-1. However, this anti-A antisera did not show reaction with any of the samples at low titration (128). An international consensus recommends that commercial ABO antisera must have a minimum of potency able to detect ABO antigens in red blood cells since the haemagglutination tests vary widely in their sensitivity and reproducibility between laboratories [23]. Our data demonstrate that variations in the concentration of anti-A and anti-B antisera belonging to the same cell clones and commercialized by different suppliers might affect the performance of haemagglutination tests.

The same serological behavior observed with the anti-A antisera and related to titration was also observed with the anti-A,B antisera. For instance, the anti-A,B antisera from mixing cell clones 9113D10 (anti-A) plus 152D12 (anti-B), reacted at the titration of 128, only with two from six evaluated samples, but reacted with five samples, at the titration of 1024. Similar reactivity was observed with anti-A,B from cell clone BIRMA-1 (anti-A) mixed with the cell clones ES-15 (anti-A), LB-2 (anti-B) and or ES-4 (anti-B). These observations support the proposition that the antibody levels of commercial antisera for blood typing seems to be important in determining their performance [22].

Our data also show that anti-A and anti-A,B commercial antisera with elevated titration increase the score of haemagglutination towards to eliminate the MF reaction. Two of the samples (C18-BD, C21-BD) presenting MF reaction at the titration 1024, showed higher agglutination score at titration 2048 when tested with anti-A from the cell clone 9113D10. One of the samples tested with anti-A,B (cell clone 9113D10 plus 152D12) presenting MF reaction at titration 128 and 256, showed strong reactivity when tested with the same antisera at the titration 1024. These observations reinforce the proposition that higher titers of antisera improve the score of agglutination, allowing the disappearance of MF reaction. The anti-A (cell clone BIRMA-1) did not react at titer 128 but showed MF at titer 1024. As this cell clone with titer higher than 1024 was unavailable we can not confirm the disappearance of the MF.

Red blood cells presenting MF with the expression weak of ABO antigens are not necessarily positive and negative. Evaluation of ABO subgroups presenting MF carried out by flow cytometry demonstrated the presence of A antigen in all red blood cells with similarities to A_x and A_2 phenotypes [25–27].

All red blood cells in MF do not react totally with low titer antisera anti-A and anti-A,B. Maybe, the small quantities of A or B antigens present a spatial distribution on the red cell membrane that does not favor the occurrence of antibody bridges between all red blood cells [28]. Therefore, antisera with high titers of anti-A and or anti-B antibodies might reach A and or B antigens spread on the red blood cell surface, reacting with them with more intensity, eliminating the MF rection. Additionally, increasing antibody concentration on the mixed anti-A,B (anti-A + anti-B) antisera modify the zeta potential, allowing red blood cell agglutination with weak expression of antigen A even in lower titers.

This study presents some limitations. Firstly, it evaluated a small number of cases. Secondly, the sequencing of the ABO exons (2 to 7) was performed for some samples and in which the alleles and genotypes were revelead. In the remaining samples, the sequencing was performed only for the exons 6 and 7. However, these limitations did not prevent us from demonstrating that low titers of antisera fail to detect the weak expression of antigen A in red blood cells.

In conclusion, the data observed in this study suggest that anti-A and anti-A,B commercial antisera with high antibody titers give better reactivity with red blood cells carrying a weak expression of A antigen.

Funding

This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo/ São Paulo Research Foundation (FAPESP 2012/07716-9 to LCM). The opinions, assumptions, and conclusions or recommendations expressed in this material are the responsibility of the authors and do not necessarily reflect the views of the FAPESP

CRediT authorship contribution statement

Marcos Paulo Miola: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - original draft. **Tatiana Elias Colombo:** Validation, Resources. **Roberta Maria Fachini:** Resources, Writing - review & editing. **Octávio Ricci-Junior:** Resources, Writing - review & editing. **Cinara Cássia Brandão de Mattos:** Funding acquisition, Resources, Writing - review & editing. **Luiz Carlos de Mattos:** Conceptualization, Funding acquisition, Project administration, Supervision, Resources, Writing - review & editing.

Declaration of Competing Interest

The authors have no conflicts of interest to disclose.

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