ORIGINAL REPORT

Mixed-field agglutination observed in column agglutination testing is not always associated with the A_3 subgroup

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Mixed-field agglutination (MFA) can be observed in forward typing of samples from A_3 individuals with serologic ABO typing methods. The results of column agglutination testing (CAT) and tube agglutination testing using different antibody clones can be discordant. In this report, we reveal our experience using polymerase chain reaction–sequence-based typing (PCR-SBT) of *ABO* exon 7 to clarify serologic method discordance of A subgroup blood typing in Northern Thai donors. A total of 21 group A blood donors with either MFA or weak agglutination on routine ABO CAT were recalled. CAT was repeated with human and monoclonal anti-A, and tube agglutination testing with monoclonal anti-A and PCR-SBT of *ABO* exon 7 was performed. A total of 13 of the 21 donors returned, and ABO CAT with human anti-A was repeated. Eleven samples showed MFA suspected to be the A_3 subgroup, and two samples showed 2+ strength suspected to be the A_{weak} subgroup. When tube agglutination testing using monoclonal antibody was performed, MFA was not observed in 9 of 11 samples with previously observed MFA from routine CAT, which were then interpreted as A_2. From PCR-SBT performed in only exon 7 of the *ABO* gene, 7 of 13 sample results were consistent with *ABO*A2* or *ABO*AW* alleles. Two samples suspected to be A_2 or A_3 had an *ABO*AW* allele. In two samples suspected to be A_{weak}, no mutation was detected in *ABO* exon 7, suggesting genetic variation elsewhere in the gene. Although other coding exons were not examined, in the alleles that could be assigned, *ABO*A3* alleles were found less frequently than would be predicted from the serologic findings. These findings suggest that when MFA in routine CAT is observed, an A_3 subgroup cannot be presumed. Caution should be exercised when MFA is noted in routine CAT. ***Immunohematology* 2018;34:49–56.**

Key Words: *ABO* gene sequencing, mixed-field agglutination, column agglutination test, discordancy, subgroup of A

ABO testing, required in blood donor processing and pre-transfusion testing, must be accurate to provide compatible blood components for transfusion to patients. If ABO discrepancy—when the red blood cell (RBC) testing result using commercial antisera (forward type) does not agree with the serum/plasma testing result using reagent RBCs (reverse type)—occurs either in donors or recipients, additional testing is needed to resolve the typing.[1,2] ABO discrepancies can be caused by technical errors or sample-specific characteristics, including weak or variant antigen expression. Technical errors should be ruled out initially. If no problems with technique (including reagents and equipment) are identified, the samples should be examined. Weak or missing reactivity, extra reactivity, and mixed-field agglutination (MFA)—characterized by distinct agglutinated cells mixed with many unagglutinated cells—can occur in both RBC and serum/plasma testing and can result in an ABO typing discrepancy. This report focuses on weak reactivity and MFA in blood group A individuals.

Possible causes of ABO discrepancies associated with weakly expressed antigens observed when the RBCs were typed with anti-A include a subgroup of A, recent transfusion, stem cell transplantation, or malignancy.[2] We investigated the expression of subgroups of A because our healthy donors had no history of transfusion, transplantation, or serious medical condition. Within the A group, A_1 and A_2 subgroups are the most common. A_1 represents about 80 percent and A_2 represents about 20 percent of group A donors.[1,2] Subgroups such as A_3, A_m, A_x, and A_{el} are found infrequently. These subgroups show weaker reactivity with anti-A reagents, mostly with human polyclonal antibody but also with monoclonal antibody, and can cause ABO discrepancies.[1] These variants result from genetic variation within the *ABO* gene producing amino acid substitutions in the glycosyltransferases.[1,3–5]

In Thailand, most blood bank laboratories perform routine ABO serologic testing using either tube agglutination testing or automated column agglutination testing (CAT). Although CAT is widely used in heavy workload laboratories such as the Thai Red Cross Society, in tertiary hospitals and university hospitals, tube agglutination testing is still the gold standard of serologic testing. Because there is no regulatory requirement regarding ABO discrepancy resolution in Thailand, the Thai Red Cross Society suggests that hospital-based transfusion service laboratories follow AABB guidelines in the interpretation of serologic reactivity and the initial investigation of the discrepancy.[1] When the discrepancy is resolved, the final ABO group, the reasons for the discrepancy, and the methods of resolution are recorded in the blood bank section of the donor record, and the donor is informed. If the

donor is determined to be a subgroup of A without any clinically significant antibodies, the blood units would be labeled with the name of the subgroup and could be transfused to patients of group A or AB or the identical subgroup. If, however, the discrepancy cannot be resolved by the hospital's blood bank by any method, then a blood sample can be sent to the Thai Red Cross Society for confirmation. Testing at the Thai Red Cross Society includes conventional tube testing for cell and serum grouping; RBC testing with anti-A,B, anti-A1, and anti-H; testing with lectins; adsorption-elution testing; and saliva testing. In some cases, samples for molecular testing might be sent out (personal communication from Department of Histocompatibility and Immunogenetics Laboratory, National Blood Center, Thai Red Cross Society). This additional testing often will delay the interpretation of the ABO group such that the blood unit will not be released for transfusion.

Serologic-based reagents should be expected to differentiate between A subgroups because weak or no agglutination can lead to wrong ABO group interpretation and the possible transfusion of a weak A subgroup to a group O recipient. This ABO-incompatible transfusion may lead to shortened survival of transfused RBCs in these recipients.[6] Correct differentiation is important for selection of blood products for routine transfusion; thus, accurate ABO typing provides transfusion safety. Moreover, differentiation of A subgroup donors is beneficial in solid organ transplant candidates. Placing a kidney from group A_2 living donors into group O or B recipients provides an excellent outcome with immediate function of the allograft and less graft rejection because of the small amount of A antigens in the donor graft. The use of A_2 donors can also expand the donor pool.[7] It has been suggested that the use of molecular testing for A_1 and A_2 subgroups be performed before elective transplantation.[7]

In our university teaching hospital, ABO typing is routinely performed by CAT and follows AABB guidelines for ABO discrepancy resolution.[1] When weak agglutination or MFA is observed, the donor's medical history is reviewed, and the sample is retested by the same CAT card type to rule out technical error. Because the degree of reactivity with monoclonal reagents can vary,[1] retesting with anti-A from a different monoclonal clone, namely one from Thai Red Cross Society with anti-A1 specificity, and with anti-H lectin is performed by tube agglutination testing. If the RBCs do not agglutinate with this monoclonal anti-A1, the A_2 subgroup might be suspected.[8] If MFA is again seen and this anti-A1 result is positive, then we presume this sample is of the A_3 subgroup.[8] When discordancy between CAT and the tube agglutination method is observed, however, the result is inconclusive. In the present study, polymerase chain reaction–sequence-based typing (PCR-SBT) of *ABO* exon 7 was used to help clarify the serologic discrepancies in those samples. Because molecular testing is not implemented in either our facility or the Thai Red Cross Society's protocol, this testing is an academic exercise attempted to resolve inconclusive serologic typing.

Materials and Methods

Sample Collection

From 1 January through 31 December 2015, we identified 21 donors with either the rare MFA pattern or only a 2+ or less reaction on ABO CAT with human antisera among all 23,918 donor blood units routinely tested at the blood bank of the Maharaj Nakorn Chiang Mai Hospital (study approval by the ethics committee at the Faculty of Medicine, Chiang Mai University; study code NONE-2558-02885). This 1400-bed,

Table 1. Primer sequences for PCR and DNA sequencing

	Primer name	Sequences (5′ → 3′)	Types	Product size (bp)
For exon 7 PCR	ABO-Ex7	CGTCCGCCTGCCTTGCAGATACG	Forward	725
	ABO-3′UTR	AGCCCCTGGCAGCCGCTCAC	Reverse	
For exon 7 sequencing	ABO-Ex7	See sequence above	Forward	
	ABO-Seq1	CGCCGCATGGAGATGATCAG	Forward	
	ABO-Seq2	TGCAAGAGGTGCAGCGGCTC	Forward	
	ABO-Seq3	TCCACCTCGCTGAGGAAGCG	Reverse	
	ABO-Seq4	CCATCATGGCCTGGTGGCAG	Reverse	
	ABO-3′UTR	See sequence above	Reverse	

PCR = polymerase chain reaction; bp = base pair.

medical-teaching, tertiary-care hospital serves the northern region of Thailand. These 21 donors were contacted and requested to return to provide an additional 20-mL sample of blood collected in EDTA tubes to repeat the CAT and perform additional ABO tests including tube agglutination testing and PCR-SBT.

ABO Phenotyping by CAT

Forward typing and reverse typing were performed using two types of gel cards: the ABO/D + Reverse Grouping card and the DiaClon ABO/D + Reverse Grouping card (Bio-Rad Laboratories, Cressier, Switzerland). The ABO/D + Reverse Grouping card contains a mixture of human polyclonal and monoclonal anti-A (cell line: A5) (referred to as "CAT with human antisera" in this report), and the DiaClon ABO/D+ Reverse Grouping card contains monoclonal anti-A (cell line: A5) (referred to as "CAT with monoclonal antisera" in this report). Testing procedures were performed following manufacturer instructions.

ABO Phenotyping by Tube Agglutination Test

Forward typing was performed by testing 5 percent RBC suspensions from each donor with monoclonal anti-A (cell line: combination of 3C4, 6G4, 16G4, and LM103), anti-B, anti-A,B, anti-A1 (cell line: 6C10), and anti-H lectin (extract of *Ulex europaeus*) (Thai Red Cross Society). RBCs were washed three times with normal saline to avoid spontaneous agglutination and nonspecific aggregation.[1] Reverse typing was also performed by testing two drops of plasma with 5 percent group A_1, B, and O cells (prepared in-house). After centrifugation, the agglutination reaction was graded, interpreted, and classified for the resulting ABO subgroups according to the *AABB Technical Manual*.[1]

ABO Gene Sequencing by PCR-SBT

Genomic DNA samples were prepared from EDTA-anticoagulated blood using the Purelink Genomic DNA Mini Kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA). PCR was performed using primers specific for exon 7 of the *ABO* gene: forward primer ABO-Ex7 and reverse primer ABO-3′UTR, both providing 725 bp PCR product (Table 1). The PCR mixture included 150 ng genomic DNA, 0.3 µmol/L gene-specific primers, 1× buffer for KOD-Plus-Neo, 0.2 mmol/L dNTP each, 1.5 mmol/L MgSO4, and 1 U KOD-Plus-Neo (Toyobo, Osaka, Japan) in 50 µL reaction volume. The cycling condition was the step-down cycle according to the KOD-Plus-Neo kit's instructions: 2 minutes at 94°C for 1 cycle, 10 seconds at 98°C and 30 seconds at 74°C for 5 cycles, 10 seconds at 98°C and 30 seconds at 72°C for 5 cycles, 10 seconds at 98°C and 30 seconds at 70°C for 5 cycles, 10 seconds at 98°C and 30 seconds at 68°C for 30 cycles, and 7 minutes at 68°C for the final extension. The PCR products were fractioned on a 1 percent agarose gel containing 0.4 µg/mL ethidium bromide and then purified using the Purelink Quick Gel Extraction Kit (Invitrogen). Nucleotide sequencing was carried out via Sanger sequencing on ABI3730XL (Bio Basic Canada, Markham, Canada) using six primers (Table 1). The Sanger contig overlapping nucleotide segments representing consensus sequences were analyzed based on information from the Blood Group Antigen Gene Mutation Database[9] and the alleles table constructed by the International Society of Blood Transfusion Red Cell Immunogenetics and Blood Group Terminology.[10]

Family Studies

Returning donors were interviewed for medical and family history. The donation history of all donors in our laboratory's records were reviewed. Their family members were encouraged to also participate in this study. After signing the consent form, a 20-mL blood sample was collected in EDTA tubes to perform ABO testing by CAT, tube agglutination testing, and PCR-SBT.

Results

Subjects Studied

During the 2015 study year, among the 23,918 routine CATs performed in blood donors at our hospital, group A donors constituted 4847 (20.3%) and group AB donors 1459 (6.1%), group B donors numbered 7343 (30.7%), and group O 10,269 (42.9%). Among the 6306 group A and AB donors, we identified 19 (0.30%) with MFA patterns, and 2 (0.03%) donors demonstrated weak (2+) expression by routine CAT with human antisera. The prevalence of ABO phenotypes in our study of Thai donors was similar to that reported in previous studies in which blood group O was approximately 40 percent, group B was 30 percent, group A was 20 percent, and group AB was 10 percent.[11–13] Among group A, the Thai Red Cross reported in 2017 that the frequency of A_3 and A_3B classified by MFA detection from tube agglutination testing was 0.04 percent and 0.46 percent, respectively.[14]

Of 21 donors who were contacted, a total of 13 (62%) returned, consented to participate in this study, and provided additional blood samples (11 with MFA patterns and 2 with 2+ agglutination). From the review of medical and donation histories, none of the donors had previous non-ABO group-

specific RBC component transfusion, ABO-mismatched stem cells or bone marrow transplants, or history of malignancy. Therefore, weak antigen expression or MFA is likely due to a genetic factor. Five of the 13 donors (donors 3, 9, and 11–13) had previously donated more than three times and had the alert message in the hospital's donation record as "suspected subgroup." The other eight donors were first-time blood donors to the center.

Discordant Results of Blood Group Serologic Tests

Table 2 shows the agglutination reaction strength of the returning 13 donors from CAT and tube agglutination testing. From routine CAT with human antisera, the 11 donors with MFA in forward typing were interpreted to be in the A_3 subgroup (donors 1–11). The two donors with 2+ agglutination strength were suspected to be of the A_{weak} subgroup (donors 12 and 13). Reverse typing of all 13 studied donors showed the typical agglutination pattern of blood group A.

The results of CAT revealed differences in the reaction patterns of forward typing between CAT with human antisera

Table 2. Serologic results of samples from 21 donors

		Forward typing					Reverse typing		
Donor ID	Test method*	anti-A	anti-B	anti-A,B	anti-A1	anti-H lectin	A_1 cell	B cell	O cell
1	CAT human antisera	mf	neg	ND	ND	ND	neg	4+	ND
	CAT monoclonal antisera	mf	neg	ND	ND	ND	neg	4+	ND
	Tube	2+mf	neg	2+mf	wk	4+	neg	4+	neg
2	CAT human antisera	mf	neg	ND	ND	ND	neg	4+	ND
	CAT monoclonal antisera	mf	neg	ND	ND	ND	neg	4+	ND
	Tube	2+mf	neg	2+mf	1+	4+	neg	4+	neg
3–8	CAT human antisera	mf	neg	ND	ND	ND	neg	4+	ND
	CAT monoclonal antisera	mf	neg	ND	ND	ND	neg	4+	ND
	Tube	4+	neg	4+	neg	4+	neg	4+	neg
9	CAT human antisera	mf	4+	ND	ND	ND	neg	neg	ND
	CAT monoclonal antisera	mf	4+	ND	ND	ND	neg	neg	ND
	Tube	4+	4+	4+	neg	2+	neg	neg	neg
10, 11	CAT human antisera	mf	4+	ND	ND	ND	neg	neg	ND
	CAT monoclonal antisera	4+	4+	ND	ND	ND	neg	neg	ND
	Tube	4+	4+	4+	neg	2+	neg	neg	neg
12	CAT human antisera	2+	4+	ND	ND	ND	neg	neg	ND
	CAT monoclonal antisera	mf	4+	ND	ND	ND	neg	neg	ND
	Tube	4+	4+	4+	neg	2+	neg	neg	neg
13	CAT human antisera	2+	4+	ND	ND	ND	neg	neg	ND
	CAT monoclonal antisera	mf	4+	ND	ND	ND	neg	neg	ND
	Tube	2+	4+	4+	neg	2+	neg	neg	neg
14	CAT human antisera†	mf	neg	ND	ND	ND	neg	4+	ND
15	CAT human antisera†	mf	neg	ND	ND	ND	neg	4+	ND
16	CAT human antisera†	mf	neg	ND	ND	ND	neg	4+	ND
17	CAT human antisera†	mf	neg	ND	ND	ND	neg	4+	ND
18	CAT human antisera†	mf	4+	ND	ND	ND	neg	neg	ND
19	CAT human antisera†	mf	neg	ND	ND	ND	neg	4+	ND
20	CAT human antisera†	mf	4+	ND	ND	ND	neg	neg	ND
21	CAT human antisera†	mf	4+	ND	ND	ND	neg	neg	ND

*Mixed-field agglutination in the tube agglutination test is distinct agglutinated cells with 2+ reaction strength mixed with many unagglutinated cells that can be confirmed under light microscope. Mixed-field agglutination in CAT is agglutinated RBCs in the upper half of the gel column with no agglutinated cells at the bottom.

†Samples from donors 14–21 were tested with only routine CAT human antisera, since they did not return to our laboratory for additional testing.

ID = identification; CAT = column agglutination testing; mf = mixed-field agglutination; neg = negative; ND = not done; wk = weak agglutination.

and CAT with monoclonal antisera. When using CAT with monoclonal antisera, only 9 of the 11 donors showed MFA (donors 1–9) and the other two showed 4+ strength (donors 10 and 11). For donors 12 and 13, weak A expression without MFA was observed by using CAT with human antisera, and MFA could be observed by using CAT with monoclonal antisera.

The tube agglutination test found MFA only in donors 1 and 2 and weak A expression only in donor 13. The other donor samples showed the expected results of the typical A blood group with strong reaction strength (Table 2). When testing RBCs with anti-A1, however, the RBCs of 11 of the 13 donors were not agglutinated by the anti-A1 and thus were re-typed by our study as the A_2 subgroup. Also, the RBCs of donors 1 and 2 were agglutinated by anti-A1 and thus re-typed as the A_3 subgroup[8] (Table 3).

Because the agglutination patterns between CAT and tube agglutination testing were significantly different, the ABO phenotypes of these 13 donors could not be concluded. We thus performed *ABO* exon 7 sequencing to aid in the prediction of the subgroup.

A Allele Sequencing Results

The *ABO* exon 7 sequencing results of each donor were compared with the Blood Group Antigen Gene Mutation Database, and nucleotide variants were identified by comparing the observed sequence in the coding sequence of *A* allele located at nucleotide 375-1065 (Table 3). Probable alleles were assigned based on the nomenclature of *ABO* alleles (Table 3). The variant sequences of all samples were found in heterozygosity. Seven samples (3–9) contained two variants, c.467C>T and c.1061delC, known to be found in multiple alleles of *ABO*A2* and *ABO*AW*. These findings rule out the A_3 subgroup. Three samples (1, 2, and 10) carry *ABO*AW31.01* or *ABO*AW31.02-05* alleles, which would be the A_{weak} subgroup. The remaining samples (11–13) had no changes in exon 7, which suggests that changes are present in other regions of the gene. Additional sequence-based typing would be necessary to assign alleles in these cases.

Discussion

In this study, we report our experience with A subgroup typing of Northern Thai blood donors in an attempt to clarify the discordant results between routine CAT and tube agglutination testing by PCR-SBT of *ABO* exon 7. Interestingly, the results from tube agglutination tests were found to correlate more with PCR-SBT. Only two samples (of 13) showed correlation between tube agglutination testing and CAT in MFA detection, whereas the remainder (11 of 13) were discordant. These 11 samples were initially identified as the A_3 subgroup from CAT with human antisera, but later were identified as the A_2 subgroup from tube agglutination testing with monoclonal antibody. Using different sources and types of antisera may have led to these discordant results. Langston et al.[15] showed that when weak A and weak B are tested by

Table 3. ABO interpretation for 13 donors by CAT, tube agglutination testing, and PCR-SBT

	CAT		Tube agglutination test		PCR-SBT	
Donor ID	Human antisera	Monoclonal antisera	Without anti-A1	With anti-A1	Nucleotides in exon 7 that differ from consensus	Probable allele (name based on ISBT allele terminology)†
1, 2	A_3	A_3	A_3	A_3	c.646T/A; c.681G/A; c.771C/T; c.829G/A	*ABO*AW.31.01, ABO*AW31.02-05*
3–8	A_3	A_3	A	A_2	c.467C/T; c.1061delC/C	*ABO*A2.01, ABO*A2.14, ABO*A2.15,*
9	A_3B	A_3B	AB	A_2B	c.467C/T; c.1061delC/C	*ABO*A2.16, ABO*AW.02, ABO*AW.03, ABO*AW.09, ABO*AW.16, ABO*AW.17, ABO*AW.18, ABO*AW.45*
10	A_3B	AB	AB	A_2B	c.646T/A; c.681G/A; c.771C/T	Suspected novel allele‡
11, 12	$A_{weak}B$	A_3B	AB	A_2B	c.467C/T	*ABO*A1.05, ABO*A1.06, ABO*A1.07 ABO*A2.10, ABO*A2.11, A215, Aw43, A312, Ax26*
13	$A_{weak}B$	A_3B	$A_{weak}B$	A_2B	None	*ABO*IVS2+3G* (A_3), *ABO*248G* (A_{weak}), *ABO*IVS1+5859C* (A_m), *ABO*52T* (A_{el}), *ABO*119A* (A_x), and *ABO*188A,189T* (A_{int})

†Because other exons of the ABO gene were not analyzed, the variant sequences found in all donors may be consistent with more than one allele.

‡Full-gene sequencing is further required to confirm the actual allele.

CAT = column agglutination testing; PCR-SBT = polymerase chain reaction–sequence-based typing; ID = identification; ISBT = International Society of Blood Transfusion.

CAT with both monoclonal and polyclonal antisera, mistyping can occur. Even when the same type of antisera (monoclonal) were used, the results were discordant between methods. This finding was most likely because the monoclonal antibodies produced by different cell lines recognize different epitopes of the A or B antigen.[16] Such discordance has not been observed in typical ABO testing, however, and several previous studies have reported the correlation between the tube agglutination tests and CAT in ABO typing.[15,17–19] From our results, CAT seems to be more sensitive than the tube agglutination method in terms of detecting MFA and weak agglutination correlated to the previous reports from other CAT systems such as AutoVue Innova and WaDiana Compact. Through the use of these systems, two-cell populations were easily observed.[20] From this point of view, there should be awareness when selecting the ABO typing method and the source of commercial antibodies to be used in a routine laboratory. Some antibody clones may not detect weak antigen expression, resulting in a different interpretation.

The importance of subgroup identification in donors is distinct from that in patients. Although the blood units from the subgroup of A blood donors without clinically significant anti-A1 or other antibodies can be transfused to typical blood group A or AB individuals, once ABO discrepancy occurs, resolution is required to identify the true blood group of such blood units before they are released for transfusion to avoid adverse reaction. Moreover, all the donor's data will then be recorded in the laboratory information system, and the true blood group will be recorded in the hospital information system. Such data will be useful for transfusion purposes if this donor returns to the hospital as a patient in the future. Note, however, that sequencing of *ABO* is not a licensed method and should be interpreted with caution, especially when incomplete, as in our cases.

ABO genotyping has not been implemented for routine use in donor centers or hospital laboratories. SBT may be useful for predicting ABO variants but is not ideal for routine ABO typing, since it is more time-consuming and expensive than serologic testing, which is simpler and more rapid.[21,22] Moreover, SBT, when it does not cover the entire coding or gene region, can result in an inability to assign alleles or an inaccurate interpretation of alleles. Additionally, predictions may be inaccurate because of alterations of gene expression unrelated to the gene regions interrogated.[21,22] In our study, we used SBT of *ABO* exon 7 as an additional test, along with additional serologic testing, to explore the genetic cause of subgroup variation and resolve ABO discrepancy.[1]

Because the majority of single nucleotide polymorphisms in the ABO coding region are located in exon 7 of the *ABO* gene and because of limited resources, we selected to examine exon 7 to identify the types of *A* allele. The cost of *ABO* exon 7 genotyping by PCR-SBT is approximately $150 per sample. In our study, PCR-SBT was performed only on samples with inconclusive results from the two accepted methods of serologic testing. Among our A subgroup donors, mutations at c.467C>T and c.1061delC of the *ABO*A2.01* allele were common, as reported in other populations.[23–25] Two group AB donors with the A_{weak} subgroup exhibited the *ABO*A1.02* allele with c.467C>T, which is common in individuals of Asian descent,[26] and one group AB donor exhibited no changes in exon 7 of the *A* allele. This finding suggests that other variants may be present in the other exons and/or introns of the *ABO* gene.

Because this study sequenced only *ABO* exon 7, we recognize that there is a limitation that genetic variation may be missed and allele assignments may be incorrect. Table 3 lists the probable allele assignment as well as other possible alleles for each sample. In samples 3–9, c.467C>T and c.1061delC were also consistent with alleles *ABO*A2.14* and *ABO*A2.15,* which share coding sequence with *ABO*A2.01,* and with alleles *ABO*A2.16, ABO*AW.02, ABO*AW.03, ABO*AW.09, ABO*AW.16, ABO*AW.17, ABO*AW.18,* and *ABO*AW.45,* which do not share coding or splice site sequence with *ABO*A2.01*. In donors 1 and 2, *ABO*AW31.02-05* allele was detected, and if exon 6 had been analyzed and c.297A>G had been found, *ABO*AW31.01* allele would be concluded. In donor 10, a novel allele was identified that is similar to *ABO*AW31.01* but without c.829G>A. No genetic variants were found in ABO exon 7 in donor 13. Based on the serology, it is possible that this donor carries one or more variants in *ABO* gene regions not interrogated by this study. Therefore, extending the sequencing to the other exons would be useful to identify the specific allele.

In conclusion, although CAT can perform blood grouping of typical ABO samples with accuracy that is comparable to tube agglutination testing, for A subgroups, CAT is more sensitive in MFA detection, but interpretation may differ from tube agglutination testing and PCR-SBT. From this study with only *ABO* exon 7 sequencing, *ABO*A3* alleles were less frequent than expected. Hence, observing MFA from routine CAT does not imply an A_3 subgroup and requires additional testing. We explored the use of *ABO* exon 7 sequencing to help clarify inconclusive results from the serologic methods;

this molecular testing can be an option in these inconclusive cases.[21,22]

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NA analyzed the data, and drafted and revised the manuscript; NA and NL designed and performed the research study; NL and PT contributed essential reagents, tools, and blood donor data; and PP and PK critically reviewed the results and revised the manuscript.

References

1. Westman JS, Olsson ML. ABO and other carbohydrate blood group systems. In: Fung MK, Eder AF, Spitalnik SL, Westhoff CM, Eds. Technical manual. 19th ed. Bethesda, MD: AABB, 2017:265–94.
2. Meny GM. Recognizing and resolving ABO discrepancies. Immunohematology 2017;33:76–81.
3. Olsson ML, Irshaid NM, Hosseini-Maaf B, et al. Genetic analysis of clinical samples with serologic ABO blood grouping discrepancies: identification of 15 novel A and B subgroup alleles. Blood 2001;98:1585–93.
4. Yip SP. Sequence variation at the human ABO locus. Ann Hum Genet 2002;66:1–27.
5. Svensson L, Rydberg L, Helberg A, et al. Novel glycolipid variations revealed by monoclonal antibody immunochemical analysis of weak ABO subgroups of A. Vox Sang 2005;89:27–38.
6. Howard PR. Basic & applied concepts of blood banking and transfusion practices. 4th ed. Maryland Heights, MO: Elsevier, 2017:103.
7. Sorensen J, Grant WJ, Belnap LP, et al. Transplantation of ABO group A2 kidneys from living donors into group O and B recipients. Am J Transplant 2001:1;296–9.
8. Tingtoy U, Sakuldamrongpanich T, Kaewmongkol R. Production of monoclonal anti-A1 blood group reagent by national blood centre, Thai Red Cross Society. Thai J Hematol Transf Med 2001;11:21–8.
9. Blood Group Antigen Gene Mutation Database (BGMUT). National Library of Medicine, ftp://ftp.ncbi.nlm.nih.gov/pub/mhc/mhc/Final%20Archive. Archived Web site originally accessed on 26 December 2016.
10. Red Cell Immunogenetics and Blood Group Terminology. International Society of Blood Transfusion. Available from http://www.isbtweb.org/working-parties/red-cell-immunogenetics-and-blood-group-terminology. Accessed 7 September 2017.
11. Nathalang O, Kuvanont S, Punyaprasiddhi P, et al. A preliminary study of the distribution of blood group system in Thai blood donors determined by the gel test. Southeast Asian J Trop Med Public Health 2001;32:204–7.
12. Fongsarun J, Nuchprayoon I, Yod-in S. Blood groups in Thai blood donors. Thai J Hematol Transfus Med 2002;12:277–86.
13. Buathong D, Hassarin S. Detection of clinically significant minor blood group antigens of blood donors in Songklanagarind hospital. Songkla Med J 2016;34:109–18.
14. Chiewsilp P, Pinyopornpanich C, Makechay S, Tubrod J, Tingtoy U, Oota S. Subgroups of A in Thai blood donors. J Hematol Transfus Med 2017;27:397–401.
15. Langston MM, Procter JL, Cipolone KM, Stroncek DF. Evaluation of the gel system for ABO grouping and D typing. Transfusion 1999;39:300–5.
16. Svensson L, Rydberg L, de Mattos LC, Henry SM. Blood group A_1 and A_2 revisited: an immunochemical analysis. Vox Sang 2009;89:27–38.
17. Tills D, Bushrod J, Ward DJ, Josef D. Typing of normal and variant red cells with ABO, Rh and Kell typing reagents using a gel typing system. Immunohematology 1991;7:94–7.
18. Williams I, Lucchesi G, Tocchetti E. An evaluation of the Diamed Microtyping System for the performance of red cell blood group serology. Aust J Med Sci 1991;12:29–34.
19. Kettler DS, Johnson MM. ABO grouping: experience with the gel test (abstract). Transfusion 1997;37(Suppl):29S.
20. Kitpoka P, Suksomboonvong P, Chongkolwatana V, Ratanasirivanich P. Comparison of microcolumn agglutination test for blood group typing and antibody screening. J Hematol Transfus Med 2014;24:7–16.
21. Reid ME. Applications and experience with PCR-based assays to predict blood group antigens. Transfus Med Hemother 2009;36:168–78.
22. Reid ME, Denomme GA. DNA-based methods in the immunohematology reference laboratory. Transfus Apher Sci 2011;44:65–72.
23. Yamamoto F, McNeill PD, Hakomori S. Human histo-blood group A^2 transferase coded by A^2 allele, one of the A subtypes, is characterized by a single base deletion in the coding sequence, which results in an additional domain at the carboxyl terminal. Biochem Biophys Res Commun 1992;187:366–74.

24. Seltsam A, Hallensleben M, Kollmann A, Blasczyk R. The nature of diversity and diversification at the ABO locus. Blood 2003;102:3035–42.
25. Hosseini MB, Helberg A, Rodrigues MJ, et al. ABO exon and intron analysis in individuals with the $A_{weak}B$ phenotype reveals a novel O^{1v}-A^2 hybrid allele that causes four missense mutations in the A transferase. BMC Genet 2003;4:17.
26. Yamamoto F, Marken J, Tsuji T, et al. Cloning and characterization of DNA complementary to human UDP-GalNAc: Fuc alpha 1----2Gal alpha 1----3 GalNAc transferase (histo-blood group A transferase) mRNA. J Mol Biol 1990;265:1146–51.

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