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ORIGINAL ARTICLE

# Investigation of Suspected Ael and Bel Phenotypes Using Mixed Agglutination Reaction With Epidermal Cells

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## Abstract

**Background/Aim:** ABO discrepancies remain an important challenge in transfusion medicine because discordant forward and reverse grouping results may require additional serological techniques and testing under different temperature and incubation conditions. Accurate investigation of such discrepancies is essential for selection of appropriate transfusion and transplantation strategies. This study aimed to investigate suspected weak A and B phenotypes in two individuals with ABO discrepancies and to assess epidermal mixed agglutination reaction (MAR) as an additional investigational method.

**Methods:** Two individuals with discordant forward and reverse ABO grouping results were investigated using routine and prolonged agglutination testing, adsorption studies, secretor-status determination, epidermal MAR, a lymphocytotoxicity assay and testing for autoimmune acid-reactive warm and cold haemolysins. Prolonged reverse grouping was performed at 4 °C and 37 °C. Adsorption studies were conducted using erythrocytes, leucocytes, saliva or mucosal cells and epidermal cells.

**Results:** In Person A, routine forward grouping showed no agglutination with anti-A or anti-B reagents, whereas reverse grouping demonstrated strong reactivity with A reagent erythrocytes and no reactivity with B reagent erythrocytes. After 1 h of prolonged incubation, the serum agglutinated A erythrocytes at 4 °C (4+) and 37 °C (2+) and B erythrocytes at both 4 °C and 37 °C (3+). Adsorption with anti-A serum reduced agglutination after exposure to the individual's erythrocytes, leucocytes, mucosal cells and epidermal cells. A blood-group substance was detected in saliva and epidermal cells adsorbed anti-A antibodies in MAR. Autoimmune acid-agglutinating antibodies were not detected. The combined serological findings were consistent with a suspected Ael phenotype. In Person K, routine forward grouping showed no agglutination with anti-A or anti-B reagents, while reverse grouping demonstrated reactivity with A erythrocytes but not with B erythrocytes. Following 1 h of prolonged incubation, A erythrocytes were agglutinated at 4 °C and 37 °C (4+ at both temperatures), whereas B erythrocytes were agglutinated at 4 °C (3.5+) and 37 °C (3+). Adsorption with anti-B serum reduced agglutination after exposure to the individual's erythrocytes, leucocytes and epidermal cells. B blood-group substance was detected in saliva and epidermal cells adsorbed anti-B antibodies in MAR. These findings were consistent with a suspected Bel phenotype.

**Conclusion:** These two cases illustrate complex ABO discrepancies that could not be resolved by routine forward and reverse grouping alone. Prolonged incubation, adsorption studies, secretor-status determination and epidermal MAR provided complementary evidence of very weak A- and B-related antigenic activity. The combined findings were consistent with suspected Ael and Bel phenotypes; however, definitive subgroup assignments were not established. Epidermal MAR may represent an additional investigational approach in selected cases of suspected weak ABO phenotypes, but its diagnostic performance and clinical utility require validation in larger studies.

**Key words:** ABO blood-group system; Blood grouping and crossmatching; Erythrocytes; Antigens, surface; Agglutination tests

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# Introduction

The ABO blood group system plays an important role in safe blood transfusion, transplantation and the management of foeto-maternal incompatibility. ABO discrepancies occur when forward and reverse grouping results do not agree. Such discrepancies require additional investigation before a definitive blood group is assigned.[1] Increasing demand for transfusion and transplantation highlights the importance of careful pre-transfusion testing.

Desai and colleagues reported 117 ABO discrepancies among 211 568 blood samples. Weak or missing antigens and antibodies, panagglutination and miscellaneous factors were identified as causes of discrepancies.[2]

Variation in erythrocyte antigen expression gives rise to ABO subgroups. Weak B phenotypes include B3, Bx, Bm and Bel. Weak antigen expression or unexpected antibodies may complicate blood-group assignment and transfusion compatibility testing.

Changes in ABH antigen expression have been described in association with malignancy. Such changes may complicate ABO grouping.[3] These cases remain challenging to resolve, since despite the appearance of highly sensitive diagnostic methods with serological and genetic technologies, they do not always allow identification of weak antigens and determination of further transfusion or donor-selection strategies. Determination of subgroups requires specialised immunohaematological techniques, namely adsorption–elution or molecular genotyping for proper detection.

Published cases of ABO discrepancy illustrate the diversity of the underlying mechanisms. One individual was typed as group O by forward grouping and as group B by reverse grouping. Repeat testing by the conventional tube technique and adsorption–elution studies demonstrated weak B antigen expression. B and H substances were detected in saliva and the findings were interpreted as a Bm phenotype.[4] A separate report described cold agglutinin interference in forward and reverse grouping in a patient with clonal B-lymphoproliferative disease.[5]

ABO-related antigen expression may differ between erythrocytes and nucleated cells. Mixed agglutination reaction (MAR) is therefore of interest as an investigational method for demonstrating antibody binding to tissue cells. Modifications of MAR have also been used to detect antisperm antibodies.[6, 7] Coombs and colleagues described mixed agglutination as a method for identifying antibody-bound cells.[8] The mixed cell agglutination test was subsequently applied to cell-surface antigens in renal cell carcinoma. Strong agglutination was associated with survival at 5.0 years in that study, suggesting a possible relationship between antigen expression and prognosis.[9] These observations provide a rationale for investigating MAR on nucleated cells in cases of ABO discrepancy, without establishing its diagnostic performance in that setting.

Previous investigations of leucocyte MAR described the use of sera from individuals without weak blood-group antigens and test erythrocytes from individuals with normal erythrocyte sedimentation rates and platelet counts.[10] The success of visualisation of adsorbed antibodies on leucocytes stimulated study of this method for blood-group testing using skin epidermis. The present study aimed to investigate ABO discrepancies using prolonged incubation, adsorption studies, secretor-status determination, testing at different temperatures and epidermal MAR.

# Methods

Two women with discrepancies between forward and reverse ABO grouping were investigated. Routine forward grouping suggested group O, whereas reverse grouping suggested group B. Erythrocytes and leucocytes were obtained from blood collected in ethylenediaminetetraacetic acid (EDTA). The cells were washed three times with 0.9 % saline, centrifuged at 1000 rpm for one minute, resuspended in 0.9 % saline and used for the investigations.

## Forward and reverse ABO grouping

Forward grouping was performed by the slide method with anti-A and anti-B reagents (*Tulip Diagnostics*, Goa, India). Reverse grouping was performed using group A, B and O reagent erythrocytes. Agglutination strength was graded from

negative (0) to strong (4+). The conventional tube technique was used for prolonged reverse grouping at 4 °C and 37 °C. Reverse grouping was also performed after serum was heated at 56 °C for 30 minutes.

## Adsorption studies

Adsorption reactions were performed at 4 °C for 12 hours in a refrigerator (*Infitek*, USA). The reactions were evaluated microscopically using a MicroMed XS-3330 microscope (China).

## Epidermal mixed agglutination reaction

Mixed agglutination has previously been described for tissue typing.[11] For the epidermal procedure used here, a 10 mg sample of dry epidermis was mixed with 3 mL of 10 % acetic acid and refrigerated overnight. The mixture was centrifuged at 3000 rpm for one minute. The sediment was placed on a glass slide and allowed to dry for 10 minutes. Then, 100 µL of 96 % ethanol was added for five minutes, followed by 100 µL of Azure-Eosin dye for 30 minutes at room temperature. The sample was rinsed with tap water. Whole polyclonal anti-A or anti-B serum (100 µL, titre 1:128) was added and the sample was placed in a humid chamber at 4 °C for 18 hours. The sample was immersed in saline for 10 minutes, transferred to fresh saline for a further 10 minutes and left at room temperature for 10 minutes. Washed A or B erythrocytes (50 µL) were added and the preparation was covered with a coverslip and incubated at room temperature for 30 minutes. The preparation was examined at ×600 magnification while the coverslip was gently tapped with a pin. Firm erythrocyte attachment that persisted during tapping was interpreted as binding to antibodies adsorbed by the epidermal cells. Three independent experiments were performed for each case, with identical results. At least 10 fields were examined by two observers.

Epidermal samples from group A individuals (four samples) and group B individuals (two samples) were exposed to anti-A and anti-B sera and monoclonal anti-A and anti-B antibodies as positive controls. Epidermal samples from group O individuals (three samples) incubated with anti-A and anti-B antibodies served as negative controls. Further negative controls consisted of group A and B epidermal samples incubated with saline or AB serum instead of specific antibodies.

## Group O epidermal cells

Group O cells lack both A and B antigens. They were incubated with anti-A and anti-B antibodies and then mixed with indicator red blood cells. No mixed agglutination was observed.

## Group A and B epidermal cells

Strong mixed agglutination was observed with the corresponding antiserum and indicator erythrocytes. These findings supported preservation of detectable antigen-related activity after tissue preparation.

## Antibody baseline control

Epidermal cells were processed through all washing and incubation phases, but saline and serum from the AB sample were used instead of anti-A or anti-B antibodies. They were then exposed to the indicator red blood cells. Complete absence of binding was observed. The indicator red blood cells remained entirely free from the epidermal cells.

## Monoclonal antibody controls

Monoclonal anti-A and anti-B antibodies were used in reagent controls. Their reactivity was checked against erythrocytes of known ABO groups. Incubation of the anti-A monoclonal antibody with known group A red blood cells produced a positive result. Contact of the anti-B monoclonal antibody with known group B red blood cells produced a positive result.

## Controls for non-specific erythrocyte attachment: wash efficiency

Two washing steps were performed after antibody incubation to remove unbound immunoglobulins. The final wash supernatant was tested against indicator erythrocytes and no agglutination was observed. This finding supported the removal of unbound agglutinating antibodies but did not, by itself, establish the specificity of every subsequent cell–erythrocyte interaction.

## Detection of warm and cold acid haemolysins

Warm and cold monophasic acid haemolysins were investigated in serum from Person A. Two rows of four test tubes were prepared as follows. Tube 1 contained 100 µL of serum and 10 µL of 1/3 N HCl. Tube 2 contained 50 µL of serum, 50 µL of complement and 10 µL of 1/3 N HCl. Tube 3 contained 100 µL of serum. Tube 4 contained 100 µL of complement and 10 µL of 1/3 N HCl. A 10 µL aliquot of a 30 % suspension of washed erythrocytes was added to each tube.

The first row was incubated at 37 °C for 60 minutes. The second row was incubated at 4 °C for 30 minutes and then at 18 °C for 30 minutes. The tubes were centrifuged at 1000 rpm for 10 minutes and haemolysis was assessed in the supernatant.

### Interpretation of the acid haemolysis assay

The following interpretation criteria were used. Absence of haemolysis in all tubes was interpreted as absence of monophasic acid haemolysins. Haemolysis in tubes 1 and 2 but not in tubes 3 and 4 was interpreted as the presence of warm and cold acid haemolysins. Haemolysis in tube 1 but not in tubes 2, 3 or 4 was interpreted as the presence of monophasic warm and cold haemolysins with weak complement activity. Haemolysis in tube 2 but not in tubes 1, 3 or 4 was interpreted as weak complement activity in the individual's serum with warm and cold haemolysins.

## Results

### Case 1: Person A

Forward ABO blood grouping of Person A showed no agglutination of erythrocytes with anti-A or anti-B reagents at room temperature, initially suggesting blood group O. In reverse grouping, the serum of Person A reacted with A erythrocytes but showed no detectable reaction with B erythrocytes at room temperature, resulting in an ABO discrepancy (Table 1).

*Table 1: Serological and adsorption findings in Person A*

| Test method | Tested sample | Reagent or indicator erythrocytes | Temperature | Incubation | Reaction strength/result |
|---|---|---|---|---|---|
| **Forward ABO grouping** | Person A erythrocyte | Anti-A reagent / Anti-B reagent | Room temperature | 10 min | 0/0 |
| **Reverse ABO grouping** | Person A serum | A / B reagent erythrocytes | Room temperature | 10 min | 4+/0 |
| **Prolonged reverse grouping (CTT)** | Person A serum | A reagent erythrocytes | 4 °C / 37 °C | 1 h | 4+/2+ |
| **Prolonged reverse grouping (CTT)** | Person A serum | B reagent erythrocytes | 4 °C / 37 °C | 1 h | 3+/3+ |
| **Reverse grouping after heat treatment** | Person A serum | A reagent erythrocytes | 4 °C / 37 °C | Serum heated at 56 °C for 30 min; subsequent incubation time 1 h | 0/2+ |
| **Reverse grouping after heat treatment** | Person A serum | B reagent erythrocytes | 4 °C / 37 °C | Serum heated at 56 °C for 30 min; subsequent incubation time 1 h | 0/0 |
| **Adsorption** | Person A erythrocytes | Anti-A serum | 4 °C | 12 h | 2.5+ → 2+ |
| **Adsorption** | Person A saliva / mucosal cells | Anti-A serum | 4 °C | 12 h | 2.5+ → 0.5+ |
| **Adsorption** | Person A leucocytes | Anti-A serum | 4 °C | 12 h | 4+ → 3+ |
| **Adsorption** | Person A epidermal cells | Anti-A serum | 4 °C | 12 h | 4+ → 2+ |
| **Adsorption** | Person A leucocytes | Anti-B serum | 4 °C | 12 h | 2.5+ → 2.5+ (no decrease) |
| **Adsorption** | Person A erythrocytes | Anti-B serum | 4 °C | 12 h | 2.5+ → 2.5+ (no decrease) |
| **Adsorption** | Person A saliva | Anti-B serum | 4 °C | 12 h | 3+ → 3+ (no decrease) |
| **Adsorption** | Person A epidermal cells | Anti-B serum | 4 °C | 12 h | 3+ → 3+ (no decrease) |

*0, no agglutination; positive values indicate graded reaction strength. CTT, conventional tube technique. Values separated by a slash correspond to the reagents or temperatures listed in the same order. Arrows indicate reaction strength before and after adsorption.*

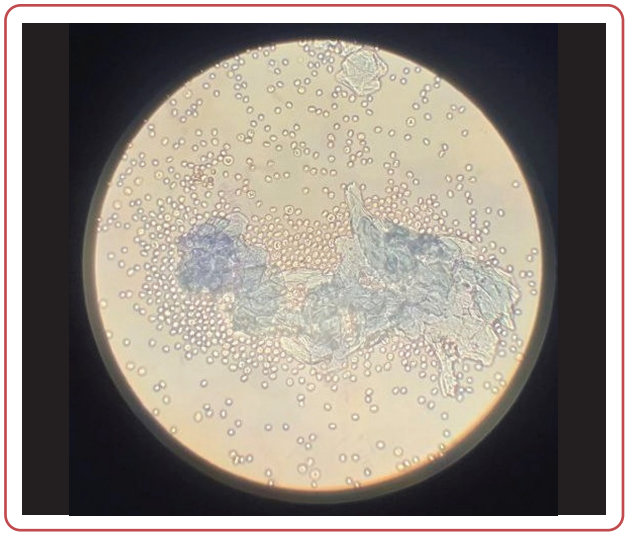


*Figure 1: Attachment of A indicator erythrocytes to epidermal cells from Person A after exposure to anti-A in mixed agglutination reaction (MAR)*

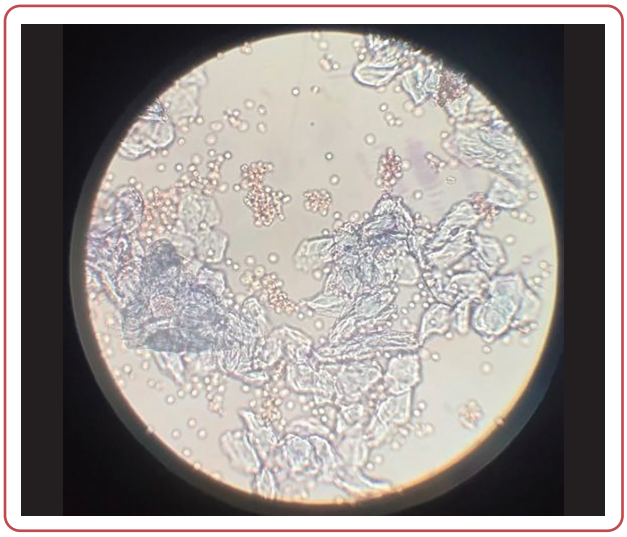


*Figure 2: No attachment of indicator erythrocytes to epidermal cells from Person A after exposure to anti-B*

Prolonged incubation in reverse blood grouping revealed additional reactivity. The serum agglutinated A erythrocytes at 4 °C (4+) and 37 °C (2+) and B erythrocytes were agglutinated at 4 °C (3+) and 37 °C (3+) after 1 hour of incubation. Thus, prolonged incubation revealed reactivity with both A and B erythrocytes that was not evident under routine room-temperature conditions.

To further characterise the temperature-dependent reactivity, the serum was heated at 56 °C for 30 minutes. After heating, the serum no longer agglutinated A erythrocytes at 4 °C but retained reactivity with A erythrocytes at 37 °C (2+). No agglutination of B erythrocytes was observed with the heated serum at either 4 °C or 37 °C. These findings demonstrate a change in the serological reactivity pattern after heating and suggest that at least part of the observed reactivity was temperature dependent.

Adsorption experiments were subsequently performed. Adsorption of the erythrocytes, leucocytes, saliva and epidermal cells of Person A with anti-B serum did not result in a decrease in agglutination. In contrast, adsorption of erythrocytes, leucocytes, mucosal cells and epidermal cells with anti-A serum resulted in decreased agglutination from 2.5+ to 2+, 4+ to 3+, 2.5+ to 0.5+ and 4+ to 2+, respectively.

Person A was identified as a secretor of A blood group substance. Epidermal cells demonstrated adsorption of anti-A antibodies and A erythrocytes were observed to attach strongly to the sensitised epidermal cells in MAR (Figures 1 and 2).

A lymphocytotoxicity assay demonstrated an inhibitory effect of serum from Person A on the lymphocytotoxicity of the control serum. Using undiluted Person A serum, lymphocytotoxicity decreased from 21 % to 16 %, whereas diluted serum (1:4 in 0.9 % sodium chloride) resulted in lymphocytotoxicity of 32 %. In a second experiment, lymphocytotoxicity decreased from 19 % to 12 % with undiluted serum and was 31 % with serum diluted 1:4 in 0.9 % sodium chloride (Table 2).

*Table 2: Effect of Person A serum on complement-dependent lymphocytotoxicity*

| Test serum | Without C′ | With C′ | With C′ and undiluted Person A serum | With C′ and Person A serum diluted 1:4 |
|---|---|---|---|---|
| Group O1 serum | 10 % | 21 % | 16 % | 32 % |
| Group O2 serum | 8 % | 19 % | 12 % | 31 % |

*C′, complement. Values indicate lymphocytotoxicity (%).*

Person A was also investigated for unexpected autoimmune acid-agglutinating antibodies. No autoimmune acid-agglutinating antibodies were detected. Weak immune anti-A and anti-B reactivity was observed. In the acid haemolysis reaction, mild haemolysis was observed with A and B erythrocytes, whereas no haemolysis was observed with the erythrocytes of Person A.

Taken together, the grouping results, adsorption findings, secretor status and epidermal MAR reactivity supported very weak A-related antigenic activity. The Ael designation remained a suspected phenotype rather than a definitive subgroup assignment.

## Case 2: Person K

In direct ABO blood group typing, the erythrocytes of Person K showed no agglutination with anti-A or anti-B antibodies at room temperature, initially suggesting blood group O. In reverse grouping, the serum of Person K agglutinated A erythrocytes but showed no detectable agglutination with B erythrocytes.

Prolonged incubation revealed a broader reaction pattern. After one hour, A erythrocytes were agglutinated by serum from Person K at 4 °C and 37 °C (4+ at both temperatures). B erythrocytes were agglutinated at 4 °C (3.5+) and 37 °C (3+) (Table 3).

Adsorption of erythrocytes, saliva and epidermal cells of Person K with anti-A antibody did not result in decreased agglutination. In contrast, adsorption with anti-B serum with erythrocytes, leucocytes and epidermal cells resulted in decreased agglutination with changes from 3+ to 0.5+, 3+ to 0 and 4+ to 2.7+, respectively.

Epidermal cells from Person K adsorbed anti-B antibodies in the MAR assay (Figures 3 and 4). The combined findings supported weak B-related antigenic activity, with Bel retained as a suspected rather than confirmed phenotype.

*Table 3: Serological and adsorption findings in Person K*

| Test method | Tested sample | Reagent or indicator erythrocytes | Temperature | Incubation | Reaction strength/result |
|---|---|---|---|---|---|
| **Forward ABO grouping (slide test)** | Person K erythrocytes | Anti-A / Anti-B reagent | Room temperature | 10 min | 0/0 |
| **Reverse ABO grouping** | Person K serum | A / B reagent erythrocytes | Room temperature | 10 min | 4+/0 |
| **Prolonged reverse grouping (CTT)** | Person K serum | A reagent erythrocytes | 4 °C / 37 °C | 1 h | 4+/4+ |
| **Prolonged reverse grouping (CTT)** | Person K serum | B reagent erythrocytes | 4 °C / 37 °C | 1 h | 3.5+/3+ |
| **Adsorption** | Person K epidermal cells | Anti-A serum | 4 °C | 12 h | 4+ → 4+ (no decrease) |
| **Adsorption** | Person K erythrocytes | Anti-A serum | 4 °C | 12 h | 2.5+ → 2.5+ (no decrease) |
| **Adsorption** | Person K saliva | Anti-A serum | 4 °C | 12 h | 2.5+ → 2.5+ (no decrease) |
| **Adsorption** | Person K erythrocytes | Anti-B serum | 4 °C | 12 h | 3+ → 0.5+ |
| **Adsorption** | Person K leucocytes | Anti-B serum | 4 °C | 12 h | 3+ → 0 |
| **Adsorption** | Person K epidermal cells | Anti-B serum | 4 °C | 12 h | 4+ → 2.7+ |

*0, no agglutination; positive values indicate graded reaction strength. CTT, conventional tube technique. Values separated by a slash correspond to the reagents or temperatures listed in the same order. Arrows indicate reaction strength before and after adsorption.*

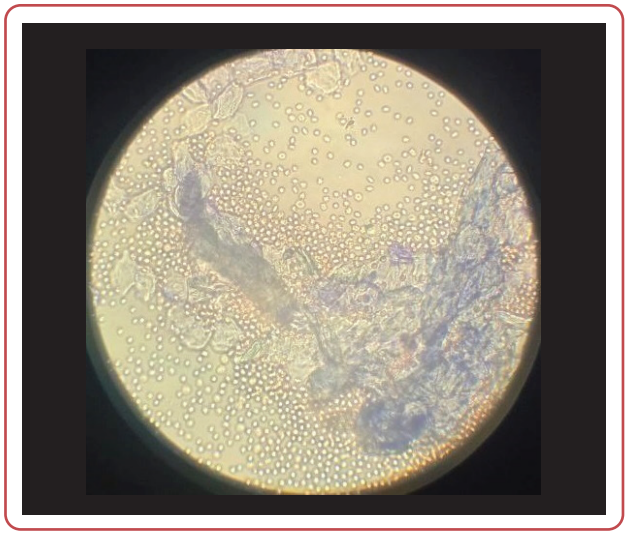


*Figure 3: No attachment of indicator erythrocytes to epidermal cells from Person K after exposure to anti-A*

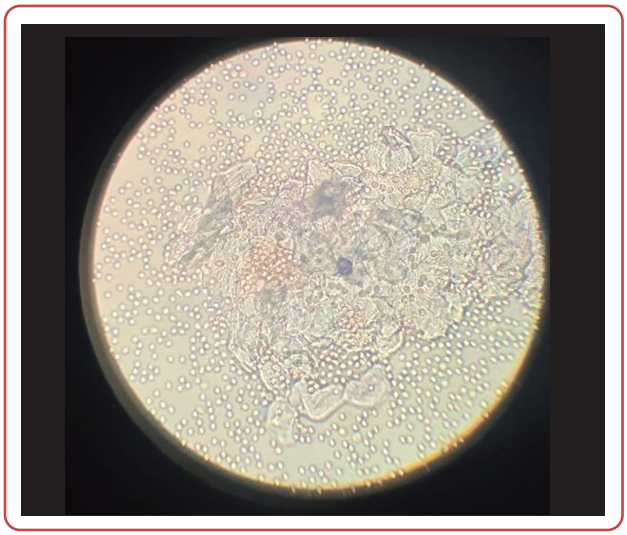


*Figure 4: Attachment of B indicator erythrocytes to epidermal cells from Person K after exposure to anti-B in mixed agglutination reaction (MAR)*

## Discussion

Accurate ABO grouping and investigation of discrepant results are essential to transfusion safety. ABO discrepancies are commonly classified into four categories: weak or missing antibodies in reverse grouping, weak or missing antigens in forward grouping, plasma or protein abnormalities and miscellaneous causes.

A published donor study reported 92 ABO discrepancies among 52 568 samples. Type 1 discrepancies were the most frequent, followed by types 2, 3 and 4. Group O was most frequently associated with type 1 discrepancies, whereas group A was more frequently associated with type 3 discrepancies.[12] A separate study from Eastern India reported an incidence of approximately 0.3 % and used additional serological investigations to resolve discrepancies.[13]

Approaches to investigating ABO discrepancies include gel-card testing, incubation at different temperatures, washing erythrocytes with warm saline, adsorption–elution and secretor studies. Gold nanoparticle enhancement of slide-test agglutination has been reported in an experimental weak B3 sample.[14] This finding does not establish performance across all weak ABO subgroups. Molecular approaches can assist with rare phenotypes, although they may not be available in every immunohaematology laboratory.

Weak ABO phenotypes represent a particular diagnostic challenge. Weak A phenotypes include A3, Ax, Aend, Am, Ay and Ael, whereas weak B phenotypes include B3, Bx, Bm and Bel. The degree of antigen expression may vary substantially and may lead to discrepancies between forward and reverse grouping.

The incidents of weak A and B blood group antigens are described in the literature. Thus, a person, initially typed as blood group O, by adsorption and saliva tests was revealed to be Aend type. The patient was a secretor of H substance. Anti-A1 was absent in the serum. [15]

Many researchers use adsorption and saliva tests after performing initial blood group testing. Thus, the person, a secretor of A and H substances did not show any presence of anti-A1 in serum, demonstrating serological characteristics of A3 type.[16]

Bel erythrocytes do not agglutinate with anti-B antibodies in forward blood group typing, making them difficult to reveal using conventional methods. The B antigens in individuals with the Bel are weakly expressed and may only be detected using sensitive methods including adsorption with anti-B antisera. Bel individuals present a unique challenge in blood typing due to weak B antigen expression, requiring careful testing and

interpretation to avoid misclassification and ensure transfusion safety.

In individuals with weak ABO phenotypes, the erythrocyte antigen density may be below the detection threshold of routine serological methods. In some cases, only a proportion of erythrocytes expresses the antigen sufficiently to produce visible agglutination, resulting in weak or mixed-field reactions. Therefore, additional techniques may be required when the routine forward and reverse grouping results are inconsistent.

Immunological visualisation of ABO-related substances on tissue and epithelial cells has also been investigated. Immunostaining studies have demonstrated ABH-related substances in epithelial tissues and changes in expression have been reported in certain pathological conditions. MAR has been used as an immunological method to demonstrate antibody adsorption on cells. In the present study, MAR was applied to epidermal cells as an additional investigational method in cases when routine erythrocyte ABO typing produced discrepant results.

Mixed agglutination has also been used to determine A, B and H blood groups in hair.[17] In tissue-based applications, indicator erythrocytes are added after exposure of the investigated cells to specific antibodies. Attachment of erythrocytes to sensitised cells provides a visual indication of antibody binding.

Sperm MAR and immunobead methods are used in antisperm antibody investigation.[6, 7] Latex agglutination has also been investigated for other purposes, including detection of *Trichomonas vaginalis* antigen.[18] These assays examine different targets and should not be treated as interchangeable evidence for epidermal ABO testing.

Previous studies demonstrated the use of MAR for blood-group antigen visualisation on leucocytes.[10] In the present study, adsorption and epidermal MAR were used to investigate two ABO discrepancies. A serum titre of 1:128 was used for adsorption and MAR; these two cases do not establish that this titre is required in all applications.

Importantly, the detection of ABH-related material on epidermal cells should not be interpreted as direct proof of the quantitative expression of ABO antigens on erythrocytes. ABO substances expressed by epithelial cells and ABO antigens expressed on erythrocyte membranes are biologically related but are not necessarily identical in their quantity, membrane organisation or detectability by a particular assay. Therefore, the epidermal MAR findings in the present study should be regarded as supportive evidence of ABO-related substance expression rather than definitive proof of an erythrocyte ABO phenotype.

Third-generation sequencing is a promising method, delivering high-confidence results. Immunohaematology centres use sequencing-based techniques to detect rare ABO blood group types. However, the application of these techniques remains difficult.[19] Despite its advantages, genotype-based matching has limitations: high costs, challenges interpreting variants, incomplete coverage of weak antigens, turnaround time constraints and limited availability of donors.[20]

Ael denotes very weak expression of A antigen on erythrocytes. Person A had no detectable agglutination by routine forward grouping, but anti-A adsorption produced reductions in reaction strength after exposure to erythrocytes, leucocytes and epidermal cells. For example, the epidermal-cell experiment showed a reduction from 4+ to 2+. The attachment of A indicator erythrocytes to anti-A-exposed epidermal cells provided visual evidence of antibody adsorption under the experimental conditions. Anti-A1 reactivity may occur in weak A phenotypes, but it must be distinguished from reactivity with general A indicator erythrocytes.

Person A showed temperature-dependent reactivity with A erythrocytes (4+ at 4 °C and 2+ at 37 °C). This pattern alone does not identify anti-A1 specificity or establish an Ael phenotype. Differentiation among weak A phenotypes requires consideration of forward-grouping reactions, salivary substances and serum antibody profiles. None of these features should be interpreted in isolation.

These findings collectively support the presence of A-related serological activity despite the absence of detectable A antigen by routine forward grouping. However, they do not provide sufficient evidence to distinguish definitively between Ael and all other very weak A phenotypes. In particular, definitive classification of Ael would ideally be supported by molecular analysis of the ABO gene or by a comprehensive combination of serological and biochemical criteria.

An Ael phenotype may resemble group O in routine forward grouping. Very weak A-related activity may require adsorption–elution or molecular investigation and serum antibody reactivity may vary. An A3 phenotype commonly shows mixed-field agglutination and positive adsorption. A and H substances may be detectable in secretors and anti-A1 may occur.

An Ax phenotype may show negative or very weak (1+) reactivity with anti-A and greater reactivity with anti-A,B. Adsorption, salivary findings and anti-A1 testing contribute to interpretation, but no single reaction pattern should be treated as universally definitive.

An Am phenotype may show no agglutination with anti-A or anti-A,B despite demonstrable A-related activity by additional testing. A and H substances may be detectable in secretors, with anti-A1 reactivity being uncommon.

An Aend phenotype may show very weak mixed-field agglutination and positive adsorption. Salivary substances and serum antibody findings contribute to differentiation from other weak A phenotypes.

Person A was interpreted as having a suspected Ael phenotype on the basis of weak A-related activity despite negative routine forward grouping. However, anti-A reactivity alone does not distinguish Ael from all other weak A phenotypes. Depending on the ABO variant, serum antibody reactivity may differ; adsorption–elution, secretor studies, specific anti-A1 testing and molecular characterisation should be considered together. Published weak A cases illustrate the need for additional testing rather than assignment from one reaction pattern.[16]

Person K showed no detectable direct agglutination with the routine reagents. Anti-B adsorption reduced reaction strength from 3+ to 0.5+ after exposure to erythrocytes and from 3+ to 0 after exposure to leucocytes. Epidermal cells reduced reaction strength from 4+ to 2.7+ and bound B indicator erythrocytes in MAR.

No change was observed in the parallel anti-A adsorption experiments. Together with the anti-B results, this supported differential antibody adsorption under the tested conditions, but did not establish 100 % analytical specificity.

Person K's serum agglutinated A erythrocytes at room temperature and also agglutinated B erythrocytes after prolonged incubation at 4 °C (3.5+) and 37 °C (3+). The additional B-cell reactivity required further antibody investigation and could not by itself establish anti-B specificity.

The combined findings were compatible with a suspected weak B phenotype, including Bel as a possible interpretation. Definitive classification was not established.

Differentiation of weak B phenotypes involves forward agglutination, salivary substances and serum antibody profiles. The reagent panel and test conditions affect interpretation.

B3 commonly shows mixed-field agglutination with anti-B. Person K showed no mixed-field reaction with the routine reagents used, arguing against the usual B3 pattern without definitively excluding all weak variants.

Bx may show weak direct agglutination, often 1+ to 2+, with selected anti-B or anti-A,B reagents. Person K showed no direct reactivity in routine testing, but the available reagent results did not provide definitive exclusion of every Bx variant.

Bm may also show negative routine forward grouping. B substance may be demonstrable in saliva from secretors and antibody profiles contribute to interpretation. Definitive differentiation from Bel requires an appropriate serological work-up and may require molecular testing.

No reduction in agglutination after exposure of anti-B to Person K's saliva was reported and was interpreted as absent or undetectable soluble B substance. Such a finding would contribute to subgroup interpretation but would not independently establish Bel.

Bm and Bel phenotypes may demonstrate overlapping serological characteristics and definitive classification may require additional molecular ABO genotyping. The additional reactivity observed during prolonged reverse grouping requires careful interpretation. Reactivity with both A and B erythrocytes, particularly when observed at different temperatures, may result from several mechanisms, including cold-reactive antibodies, autoantibodies, nonspecific serum reactivity, complement-dependent effects or antibodies with unusual temperature characteristics. Consequently, the presence of additional

agglutination after prolonged incubation cannot automatically be attributed to an ABO subgroup.

In Person A, reactivity was observed with A erythrocytes at both 4 °C and 37 °C and with B erythrocytes at both temperatures. The investigation did not demonstrate autoimmune acid-agglutinating antibodies, although weak immune anti-A and anti-B reactivity was observed. Mild haemolysis was also detected in the acid haemolysis reaction with A and B erythrocytes but not with autologous erythrocytes. These findings indicate that the serum contained additional serological reactivity that could influence interpretation of the reverse-grouping results.

Importantly, in Person A the serum showed decreased activity of anti-A antibodies at 37 °C; in Person K the serum showed decreased activity of anti-B antibodies at 37 °C. The heating experiment provided additional information concerning the temperature dependence of this reactivity. Heating the serum of Person A at 56 °C for 30 min eliminated the reaction with A erythrocytes at 4 °C but did not eliminate the reaction at 37 °C. Reactivity with B erythrocytes was no longer observed after heating. Thus, the experiment demonstrated that different components of the observed serum reactivity had different thermal characteristics. However, this experiment alone does not identify the specific antibody responsible for the reactions and should therefore be regarded as a supplementary serological observation rather than a diagnostic criterion for Ael.

Unexpected reactions with A or B indicator erythrocytes require differentiation from autoantibody-related interference.[21] The present investigation included acid haemolysin testing. A previous study of ABO discrepancies considered serum-related interference with haemagglutination.[22] Leukaemia-associated weakening or loss of ABO antigens has also been reviewed.[23]

Antibody specificity and temperature dependence are important considerations in the investigation of unresolved ABO grouping. A published case with strong anti-A and mixed-field anti-B forward reactions, but no reverse-grouping reaction, was resolved by polymerase chain reaction with sequence-specific primers. A BA1 genotype was reported and interpreted as an A1B phenotype.[24] The lymphocytotoxicity experiment demonstrated an inhibitory effect of Person A serum on lymphocytotoxicity under the experimental conditions used.

The lymphocytotoxicity results provide an additional observation concerning serum activity in ABO discrepancy. Serum-related inhibition of typing antisera has been described in other investigations.[22, 25] In the present experiments, undiluted serum reduced measured lymphocytotoxicity, whereas diluted serum produced higher values. The assay was complement-dependent, but these observations did not identify a specific complement-inhibitory factor or establish protection from autoimmune haemolysis. Such interpretations would require additional experimental characterisation.

Malignancy-associated ABO discrepancies have been reported. Plasma cell myeloma may be associated with altered grouping reactions.[26] Transient weakening of A-antigen expression and subsequent reappearance have also been described in acute myeloid leukaemia. In one reported investigation, initial plasma reactivity with A1 erythrocytes (2+) and B erythrocytes (4+) was followed by reappearance of A antigen, with molecular analysis supporting an AA genotype.[27]

## MAR as an additional investigational method

The present study explored the use of epidermal MAR to investigate weak ABO-related substance expression in two cases with ABO discrepancies. The observation of A erythrocyte attachment to epidermal cells from Person A after anti-A exposure and B erythrocyte attachment to epidermal cells from Person K after anti-B exposure, provides evidence that the investigated epidermal cells were capable of adsorbing the corresponding antibodies under the experimental conditions.

MAR positivity should not be interpreted as a direct substitute for conventional erythrocyte ABO typing. The method detects antibody adsorption by epidermal cells and therefore provides information about ABO-related substances on those cells. It does not directly measure the amount or functional expression of ABO antigen on circulating erythrocytes.

This distinction is particularly important for weak ABO phenotypes. The relationship between epithelial ABH substance expression and erythrocyte ABO antigen expression may be influenced by secretor status, tissue-specific expression and the biochemical properties of the ABO antigens. Therefore, epidermal MAR should be considered

an adjunctive method that may provide additional information when conventional serological testing is inconclusive.

The specificity of the method required appropriate controls. The study included group O, A and B epidermal cells as controls for the attachment of indicator erythrocytes. Such controls allowed the specificity of erythrocyte attachment to be distinguished from nonspecific cellular adhesion.

### Relationship between MAR and adsorption testing

Adsorption remains an established approach for the investigation of very weak ABO antigens. In contrast to adsorption, MAR provides visualisation of antibody adsorption directly on the investigated cells through attachment of indicator erythrocytes. This visual characteristic may potentially provide complementary information concerning the distribution of antigen-related structures on individual cells.

However, MAR should not be considered a replacement for conventional adsorption-elution testing. The two approaches investigate related but not identical aspects of antigen–antibody interaction. Future investigations should therefore compare the results of epidermal MAR with standard adsorption–elution, serological subgrouping, secretor studies and molecular ABO genotyping. Such comparisons would help establish the clinical and diagnostic significance of epidermal MAR.

### Transfusion implications

An unresolved ABO discrepancy has implications for recipient compatibility testing and donor-unit disposition. Epidermal MAR findings alone do not establish a definitive ABO group for clinical component selection.

Component selection must take account of the complete serological investigation, antibody specificity and the urgency of transfusion. Where urgent transfusion is required before resolution, compatible group O erythrocytes may be considered under the transfusion service's established procedures.[2] This does not mean that every patient with an unresolved discrepancy can be assigned a definitive group O phenotype or that all haemolytic risks are eliminated.

Plasma and platelet selection must be assessed separately under local transfusion-medicine policy. The present two-case investigation does not establish a universal requirement for group AB plasma or platelets.

Very weak donor antigen expression may be missed by routine testing, so discrepant donor results require further investigation. The risk cannot be inferred solely from epidermal MAR positivity. A donor unit with unresolved discrepant ABO results should not be issued as though its blood group were established.[2]

A suspected Ael or Bel interpretation does not justify automatic relabelling as group A or B or a claim of unrestricted safety for recipients of that group. Definitive donor classification requires resolution through the appropriate transfusion-service procedures.

### Limitations of the study

Molecular ABO genotyping was not performed. The results supported suspected weak A and weak B phenotypes, with Ael and Bel remaining unconfirmed subtype interpretations. The study included only two cases. Consequently, the findings cannot be used to establish the diagnostic sensitivity, specificity, reproducibility or clinical utility of epidermal MAR. The relationship between ABO-related substances detected on epidermal cells and ABO antigen expression on erythrocytes requires further investigation. MAR positivity demonstrates antibody adsorption by epidermal cells and does not directly quantify erythrocyte ABO antigen expression.

## Conclusion

The two cases presented demonstrate complex ABO discrepancies, when routine forward and reverse blood group typing produced discordant results. Additional serological investigations, including prolonged incubation, adsorption studies, secretor status determination and epidermal MAR, provided evidence of very weak A- and B-related antigenic activity. The findings were interpreted as compatible with suspected Ael in Person A and suspected Bel in Person K, but did not establish definitive subgroup assignments. Epidermal MAR may provide supplementary information on ABO-related substances in discrepant cases. Its diagnostic performance and clinical utility require further investigation.

## Ethics

The study was approved by the Kharkiv National Medical University Ethics Committee (protocol No 10, dated 4 May 2025). Written consent was obtained from the participants.

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## Conflicts of interest

The authors declare that there is no conflict of interest.

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## Data access

The data that support the findings of this study are available from the corresponding author upon reasonable individual request.

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