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Research Article

Absence of 1061C deletion in A2 blood subgroup validated through gene sequencing in the Malaysian population

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Abstract

Introduction: ABO blood grouping is an important antigenic blood typing tools in blood transfusion and organ transplants. Mismatching of blood during transfusion would lead to undesired transfusion reactions. Due to rare occurrence of rare blood group such as A_2 subtype, regular blood grouping technique would have missed the identification of blood group. **Objectives:** In this study, the identification of A_2 subgroup using routine serological technique was validated via DNA sequencing technique. **Materials and Methods:** A total of 656 students participated in this study consist of Malay (87.0 %), Chinese (0.4 %), Indian (11.4 %) and others ethnic group (0.9%) respectively. Monoclonal antisera A, B, AB, D, A_1 lectin and H lectin were used to identify the antigen on red blood cells. DNA sequence analysis was applied to examine single nucleotide polymorphisms (SNPs) at position 467 (substitution of C>T) and 1061 (deletion of C) on coding region of ABO gene. **Results:** Our findings showed of 656 blood samples, 256 (39.0%) were blood group O, 190 (29.0%) were blood group B, 179 (27.3%) were blood group A and 31(4.7%) were blood group AB. The frequency of A_1 subgroup is 177 (99.0%) and A_2 subgroup is 2 (1.0%). From 179 A blood group, only 2 samples showed negative reaction towards anti-A_1 lectin. DNA sequence analysis revealed the SNPs at nucleotide 1061 position in sample 2, however this mutation was absence in sample 1, suggesting presence of another mutation that may result in the A_2 phenotype. **Conclusion:** The current study reported the absence of 1061C deletion in A_2 blood group sample among Malaysian population.

Keywords: ABO bood-group system; A_2 subgroup; single nucleotide polymorphisms; DNA sequencing; blood donation

1.0 Introduction

Safe blood transfusion was made possible through the discoveries of ABO blood group in the 1900. By performing a series of mixing experiments with the red blood cell (RBC) and serum of his colleagues, Karl Landsteiner first identified three major blood group, A, B, and O. A year later, the less frequent AB blood group was included as the fourth blood type. For that, he received the Nobel Prize in physiology and medicine in 1930 for his work (Schwarz, 2003).

The blood groups were the results of the presence or absence of naturally occurring antigen on the surface of RBCs as well as antibodies against them in the serum. For example, RBCs of group A will agglutinate with serum of blood group B due to the presence of antigen A on the RBCs of blood group A and antibody A in the serum of blood group B. Similarly, RBCs of group B will agglutinate with serum of blood group A. As a result, A blood donor cannot transfuse their RBCs into the B blood recipient serum.

The O blood group signifies the absence of both A and B antigen on the surface of the RBCs but presence of both A and B antibodies in the serum. This enable blood O individuals to become a universal donor but unfortunately can only receive blood from the same group. Conversely, blood group AB indicates the presence of both A and B antigen on the surface of the RBCs but absence of both A and B antibodies in the serum making them the universal recipient but not a universal donor. Together, the ABO blood group discovery formed the very foundation of transfusion medicine (Manoharan, 2013).

A more recent work has led to the discovery of subgroups of blood type particularly A group, one of which was exhibiting weaker expression of the A antigen, which was categorised as A_1 and A_2 (Giriyan et al., 2017). There are also other subgroups such as A_3, A_{end}, A_{finn}, A_x, A_m, A_{el}, based on the magnitude of the reactivity of the RBCs with the A antibodies. Individuals with subgroup A_1 made up of 80% of the whole blood group A population. Among the subtypes of A, A_2 is more commonly found within the remaining 20% of the blood group A population (Giriyan, Agrawal, Bajpai & Nirala, 2017; Mahapatra, Mishra, Sahoo & Sahoo, 2016, Elnour et al., 2015).

At young age, individuals with A_2 blood were not conventionally identified until they developed an antibody towards the A_1 cells. This antibody could develop from an exposure (sensitisation) towards A_1 antigen by either blood transfusion or pregnancy.

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Once an A_2 blood has developed an antibody against A_1, the A_2 can only be transfused with A_2 blood or with O blood that is compatible (Padmasri, Bhatara & Iyengar, 2014). Therefore, misidentifying the blood group can be lethal.

Blood groups are genetically influenced, where parents pass down their blood group allele to their offspring. The ABO gene is located at chromosome 9 and it encodes for three types of alleles that are known as A, B and O allele (Westhoff & Sloan, 2008). The A_2 phenotype have been associated with the presence of A201 allele, an A allele variant that is characterised by the two Single Nucleotide Polymorphisms (SNPs), at nucleotide 467 (substitution of C to T) and nucleotide 1061 (deletion of C), within the exon 7 of ABO gene (Liu, Li, Mao & Hu, 2013).

Routine blood typing usually requires multi-step process to specifically identify the A_2 blood group. In this study, screening of A_2 subgroup via routine serological technique was validated using genetic sequence analysis that identify SNP at nucleotide 467 and 1061.

2.0   Materials and methods

2.1   Ethical approval

This research has been approved by the Ethic Committee of the Faculty of Engineering and Life Science UNISEL (Ref: J160170E).

2.2   Sample size calculation

The sample size was determined by applying prevalence study formula with 95% level of confidence and 0.06 precision (d). According to Musa et al. (2012), the prevalence of blood group A individual in Malaysia is about 26.9%. Hence, by substituting the prevalence data, at least 160 blood group A samples need to be obtained for this study.

2.3   Study design

Participants were recruited among new student intake of Universiti Selangor (UNISEL) in the year of 2017. A total of 656 respondents with informed consent was obtained from students attending the health screening day to draw 1 ml of whole blood. Venepuncture was performed by the certified phlebotomist and 1 ml of blood was collected in EDTA tube. All blood samples were stored at 4ºC for 14 days before they were discarded.

2.4   Inclusion and exclusion criteria

All students that attend the UNISEL health screening day and provide informed consent was included in the study. Students who attend the health screening day but refuse to provide informed consent were excluded from the study.

2.5   Blood group screening

The ABO blood grouping was performed on tile using monoclonal anti-A and B. The two antibodies were placed separately on the tile with label. Blood samples from study participant were dropped onto the antibodies and were mixed with application stick. The presence and absence of agglutination was observed and the blood group was identified. The blood identified as group A was further evaluated using anti-A_1 lectin. The antibody was placed on a tile and blood sample was dropped onto the antibody. The presence and absence of agglutination was observed and participants were classified as A_1 or A_2 subgroup.

2.6   Serological confirmation

Blood group A sample that tested negative with anti-A_1 lectin was reconfirmed by performing full blood grouping including the forward, reverse, A_1-lectin detection, and antigen H detection using standard tube method. The monoclonal anti-A, anti-B, anti-AB, anti-D were used in forward method while the manually prepared A, B and O known cells used for reverse grouping. For A sub grouping, anti-A_1 lectin (Dolichos biflorus) were used. H antigen were identified using anti-H lectin (Ulex europaeus).

2.7   DNA sequencing

The A2 group blood confirmed from the serological method were further examined using DNA sequencing to identify the single nucleotide polymorphisms at nucleotide 467C>T and 1061del C of A transferase. The allele reported to contain these two SNPs is the A201 allele. Exon 6 and 7 of ABO gene were partially sequenced where these two mutations are located. The position of mutation was determined using reference SNP (rs) number which obtained from SNPedia. The referred rs number are rs1053878 (467C>T) and rs8176750 (1061delC) of ABO gene. The sequence of this rs number were obtained from National Centre for Biotechnology Information (NCBI). *tical analysis*

2.8   Statistical analysis

Relationship between ethnic group and blood group distribution was evaluated using Chi square analysis. Data was analysed using Statistical Package for the Social Sciences (SPSS) software version 16. The frequency of ABO blood group and A subgroup were analysed using SPSS, V25.

3.0   Results

A total of 656 new intake students participate in this study. The study participants consist of 468 (71.3%) females and 188 (27.8%) males. The participants ethnic groups include Malay, Chinese, Indian and Other ethnic group with frequency of 571 (87.0%), 4 (0.4%), 75 (11.4%) and 6 (0.9%) respectively. In terms of blood groups distribution, 256 (39.0%) were blood group O ,190 (29.0%) were blood group B, 179 (27.3%) were blood group A and 31(4.7%) were blood group AB. The relationship between the ethnic group and the blood group were determined using Chi-square analysis. Ethnic group were found to have no significant relationship with the blood group as depicted in Table 1.

Table 1: Distribution of ethnic group and blood group of students in UNISEL.
No significant relationship was found between the ethnic group and blood group distribution.

Ethnic Group	Malay	Chinese	Indian	Other	Chi-square	p-value
N (%)	571 (87.0%)	4 (0.4%)	75 (11.4%)	6 (0.9%)		
Blood Group	A	B	O	AB	0.474	8.615
N (%)	179 (27.3%)	190 (29.0%)	256 (39.0%)	31 (4.7%)		

Samples of the 179 (27.3%) blood group A were tested further with anti-A_1 lectin. The frequency of the A subgroups was found to be 177 (99%) and 2 (1%) for A_1 and A_2 subgroup, respectively. The distribution of blood groups as well as the A subgroups were illustrated in Figure 1.
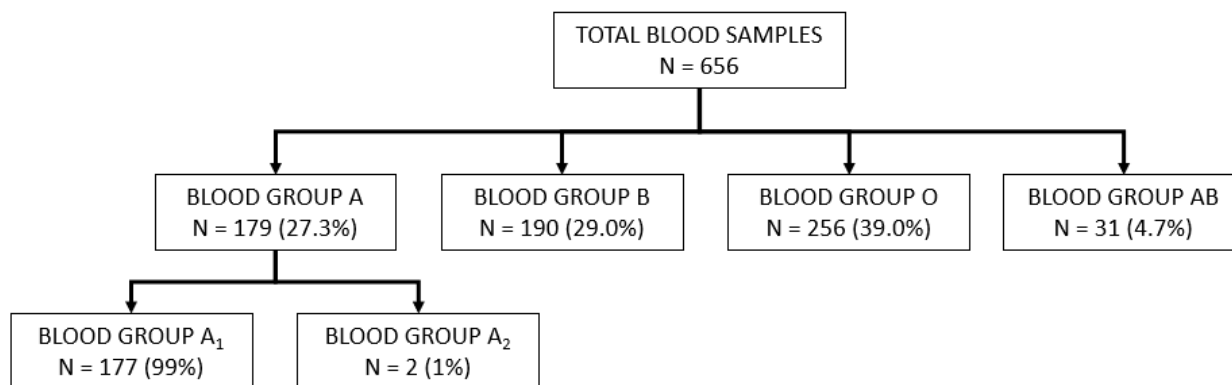

Figure 1: Blood group A subgroup distribution. Blood group A_2 was determined using anti-A_1 lectin.

The two samples of blood group A that were negative with anti-A_1 lectin were selected for further test and the sample are labelled as sample 1 and sample 2. The result of forward and reverse blood grouping, anti-A1 lectin, and anti-H antigen detection for sample 1 and 2 are presented in Table 2.

Table 2: Agglutinated grades were gradually increased from (+) to (4+). Serologic reaction between sample 1 and 2.

Sample	Forward grouping			Rhesus	Reverse grouping			Lectin	
	Anti-A	Anti-B	Anti-AB	Anti-D	A-RBC	B-RBC	O-RBC	Anti-A1	Anti-H
1	2+	0	2+	4+	0	4+	0	0	4+
2	4+	0	4+	4+	0	4+	0	0	4+

The forward and reverse grouping were tally for both samples. Sample 1 showed weak reaction with anti-A and anti AB while sample 2 reacted strongly with anti-A and anti-AB. Sample 1 and 2 were sent for DNA sequencing and the results were shown in reverse complement arrangement. The genetic sequencing revealed that only sample 2 has the 1061C deletion (Figure 2) while sample 1 did not have the expected mutation (Figure 3). This suggests the possibility of other mutations present in this allele which caused the negative reaction with anti-A_1 lectin. Nevertheless, substitution of 467C to 467T nucleotide were detected in both samples.

4.0   Discussion

The ABO blood group is crucial in transfusion medicine. Distribution of the blood groups among Malaysian population has been reported previously (Manoharan, 2013; Musa et al., 2012). In this study, the findings of highest and lowest frequency of blood group O and AB, respectively, agree with the findings from the previous study (Manoharan, 2013). However, the second-most frequent blood group in UNISEL population is group B as compared to group A reported in the previous study (Musa et al., 2012). Both studies utilize serological technique to identify the blood group.

In different populations around the globe, variants in terms of blood group distribution are apparent. Regardless, blood group O is oftentimes reported as the most prevalent blood group (Agrawal et al., 2014; Manoharan, 2013; Musa et al., 2012). However, the blood group A has been reported to have a higher prevalence in Central and Northern Europe (Dean, 2005). Blood group AB is the lesser reported group in almost all population. Since the blood group is genetically dependent, variation in a particular population can be due to factors such as migration, mutation and marriage between different ethnic groups (Amin, Susanto & Naher, 2014).

In this study, majority of the participants are of Malay ethnic group with a frequency of 571 (87%) out of 656 participants. The remaining 85 participants are Indian, Chinese and Others ethnic group. The Indian ethnic group has the second highest frequency of 75 (11.4%). The Chinese ethnic group carries the least frequency of 4 (0.4%). Any other ethnic besides these three major group are classified as Others group. The Others ethnic group frequency is 6 (0.9%). The participant of different ethnic group was not adequately proportioned because the
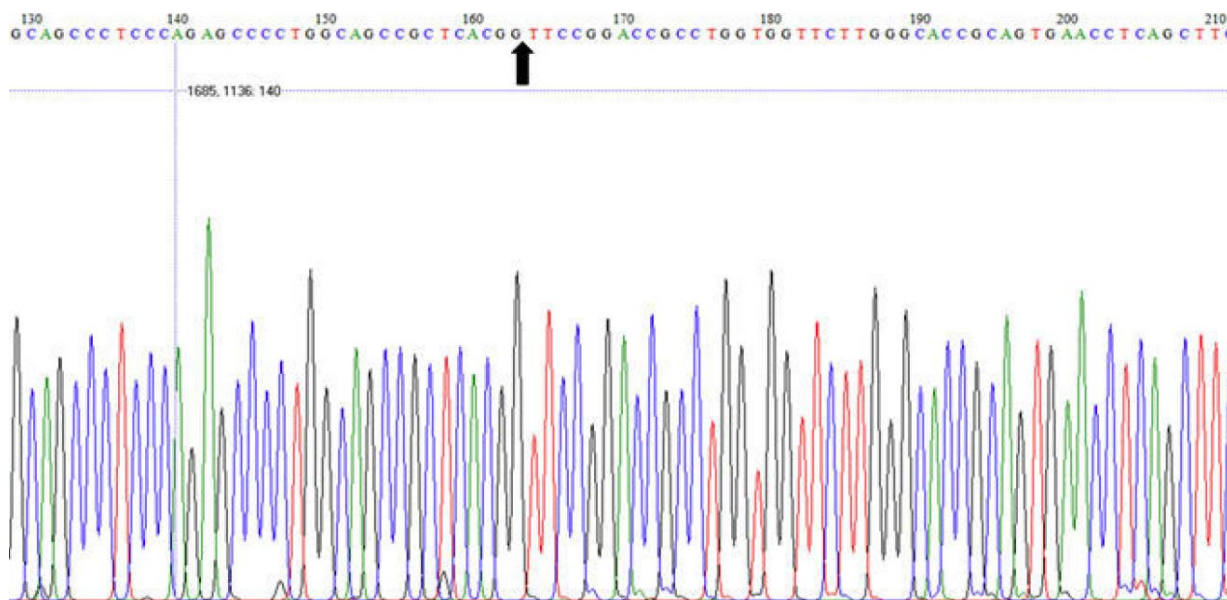
Figure 2: Partial DNA sequencing chromatograms of ABO gene of A_2–related alleles identified by direct sequencing. The arrow indicated the deletion of 1061C nucleotide at rs 8176750 of Sample 2.
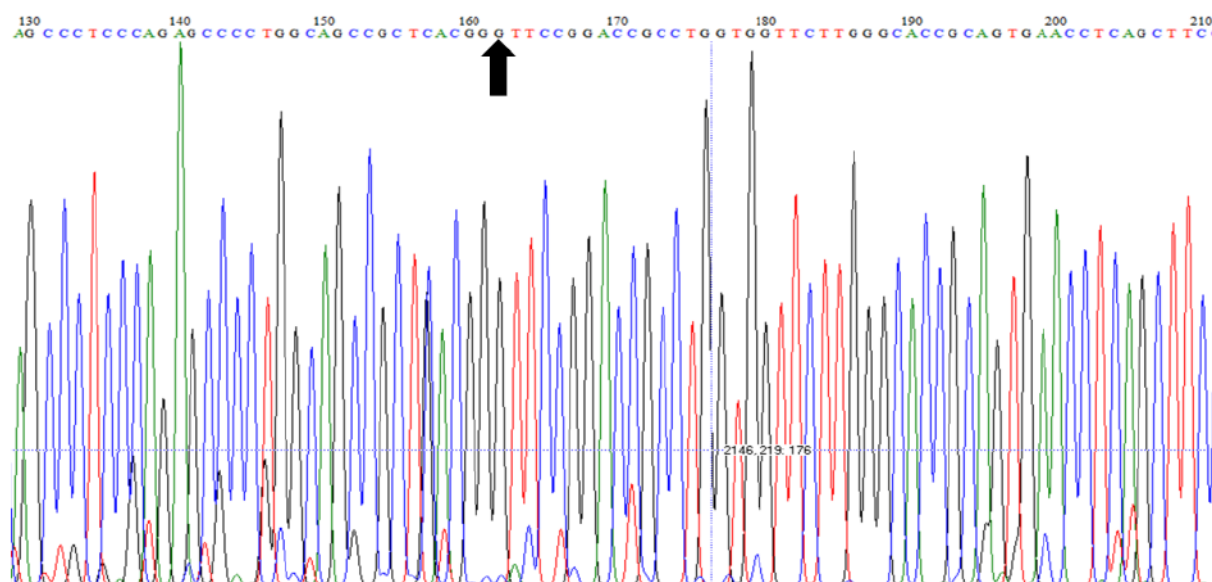

Figure 3: Partial DNA sequencing chromatograms of ABO gene of A_2–related alleles identified by direct sequencing. In sample 1, the arrow indicated the 1061C nucleotide at rs 8176750 that was not deleted.

participant was randomly selected among the new students. In future study, sample size calculation should be performed to identify the number of participants from each ethnic group to precisely determine the association of blood groups and ethnic groups.

From the 656 participants, only 179 of them were blood group A. The 179 blood group A samples were sub- grouped into A_1 and A_2 based on their reaction with anti- A_1 lectin. Out of 179 sample, only 2 blood group A samples showed a negative reaction with anti-A_1 lectin and these group were categorised as A_2. These reactions indicated the absence of A_1 antigen on RBC. Besides that, 177 blood group A samples agglutinate with anti-A_1 lectin due to the presence of antigen A_1 on the RBC surface. From these results, we can conclude that A_1 is the common A subgroup while the A_2 is the rare A subgroup. A similar study was carried out in North Karnataka region and the frequency of A_1 is 98.90% and A_2 is 1.10% (Giriyan et al,2017). Another study in White Nile Sudan reported the frequency of A_1 in their study area is 93.42% and A_2 is 6.58% (Elnour et al., 2015).

Landsteiner's law describes that the human body produces an antibody against the antigen that they do not possess. These antigen and antibody combinations prevent the self-destruction of own RBC. The blood group antibodies can be grouped into induced and naturally occurring. The induced antibody can be defined as a type of antibody that is produced in response to specific antigen and usually occur during transfusion, transplantation or pregnancy (Padmasri et al, 2014). The production of naturally occurring antibodies remains unknown and was associated with early life infection.

Fortunately, with the occurrence of natural antibodies, blood group test can be verified via the forward and reverse method. In the forward test, a known antibody is used to detect antigen on the RBC while in reverse test, RBC with known antigen is used to detect the antibodies in the sera. The subgroup test is performed when forward and reverse grouping do not give the same result or genetic inconsistencies are found (Kochhar, 2012). The phenotype which has a different amount of A and B antigen on red cell and secretion are known as a subgroup.

Generally, the major subgroup of A, A_1 and A_2 was reported to have better reactivity with antibody A (anti-A) while other minor subgroups of A showed a weak reaction with anti-A (Mahapatra et al. 2016). In this study, sample 1 resulted in a weak reaction (2+) with monoclonal anti-A while sample 2 give strong agglutination reaction (4+). Based on the reaction with anti-A, sample 1 probably belongs to the other minor subgroup of A.

The A_1 and A_2 subgroups can be distinguished through reaction with anti-A_1 lectin. The anti-A_1 lectin only reacts with A_1 RBCs. On the other hand, A_2 phenotype has abundant of H antigen on the surface of RBCs compared to A_1 phenotype. The higher level of H antigen among A_2 subgroup is due to the lack of enzymatic reaction that involved in the formation of A antigen from H antigen. In addition, the level of H antigen is higher among blood group O individual because the blood group O does not possess the enzyme that changes the precursor H into A or B antigen. In this study, blood group O was used as the positive control in H antigen detection. The blood group AB also can be sub-grouped into A_1B and A_2B, and A_2B blood group appeared to be slightly higher in frequency compared to A_2 alone. This phenomenon is probably due to the strong action of the B gene that decreased the production of A antigen on RBC surface (Mahapatra et al., 2016).

The A2 subgroup was first discovered by Yamamoto et al. in 1992 as A201 allele mutations which consist of substitution of 467C>T (Pro156Leu) and a single C deletion in a series of three C's at nucleotide 1059 to 1061 (1060delC) (Yamamoto, McNeill & Hakomori, 1992). These findings have been expanded over the years with better precision techniques. According to Blood Group Antigen Gene Mutation (BGMUT), currently there are about 179 of ABO alleles reported worldwide (BGMUT, 2017). In the case of A_2 subgroup, there are 15 alleles that are responsible for A_2 phenotype (BGMUT, 2017). Allele A201 is predominant among Caucasian while A202, A203, A204, and A206 are common in the Japanese population (Yip, 2002a). Different A_2 alleles have different mutations and polymorphisms among the nucleotides. A202 allele has substitution at 1054 C>T, A203 at 1054 C>G, A206 at 1009 A>G and etc.

Our study has identified one SNP at nucleotide position 1061 with single deletion of C nucleotide of A201 background allele from sample 2 without substitution of 467 C>T. In regards to our findings, sample 2 would probably be best identified- as A205 allele as described by Olsson & Chester in 1996 and Yip in 2000 (Olsson & Chester, 1996, Yip, 2000b). However, sample 1 without both of these mutations would probably belong to the other A_2 alleles or another subgroup of A such as A_3, A_w, A_e, A_m. This irregularity has made us speculated that sample 1 would probably a newly discovered A2 alleles. However, further investigation is needed to confirm our suspicion.

The coding regions of ABO alleles are located at chromosome 9q34.1-q34.2 that consist of 7 exons. Alterations involved in this region such as single base substitution or single base deletion were accounted for the variability of these three alleles. Usually, most of the base alterations in the coding region occurred in exon 6 and 7. Different ABO alleles would produce different transferase enzyme that specifically determine their blood phenotype. An allele would produce A transferase, B allele for B transferase, whereas O allele would end up with the absence of the transferase enzyme. The transferase is responsible for the enzymatic modifications of the H antigen, the precursors for both A and B antigen. A_1 alleles are responsible for the translation of A transferase enzyme which modifies H antigen to A antigen. Mutations alongside these regions would therefore result in its reduced or altered enzymatic activity that resulted in for other subgroups of A such as A_2. This could be explained through our serologic testing whereby A_2 blood (sample 1 and 2) did not react towards anti-A1 lectin. Sample 2 which has single nucleotide deletion at 1061 (1060delC) would probably cause a frameshift that produce an additional of 21 amino acids at the carboxyl terminus of the transferase enzyme resulting in its altered enzymatic activity.

Although the A subgroup occurrence is rare, reports on its transfusion complications have been documented (Padmasri et al., 2014). Naturally, the anti-A_1 antibody is not detected in the A_2 individual's serum, however, it is present if they have developed an antibody to A_1 cells through A_1 transfusion sensitization or pregnancy. Once A_2 blood group patients have developed an antibody against the A_1 antigen, the A_2 can only be transfused with A_2 blood or with compatible O blood. In the current study, individuals carrying A_2 subgroup blood has been identified from the multi-step routine blood typing protocol using forward and reverse grouping with anti-A_1 lectin reaction.

Awareness of rare blood groups and subtypes will help in improving inventory management in the blood bank. Development of better techniques to identify rare blood type can help shifting the focus to recruiting minority donors to increase the pool of rare blood in the blood bank. Most of the time, identification of rare blood groups is coincidental via routine pre-transfusion testing or pregnancy follow-up, provided that the antibodies corresponding to the rare specificity are present.

There is a growing awareness of the impact of the genomics technology on transfusion medicine and its potential to transform the way blood is selected for transfusion. Single-nucleotide polymorphisms (SNP) genotyping for blood could offer an alternative to the current antibody-based technology. To our knowledge, this is the first report of using single nucleotide polymorphisms to identify A_2 subgroup in the Malaysian population.

5.0   Conclusion

In this study, we demonstrated the utility of gene sequencing technique in identifying A subgroup blood type, particularly the A_2 subtype to validate the serological findings. We reported the absence of 1061C deletion, a common SNP in A_2 blood phenotype, among Malaysian population blood samples.

6.0   Acknowledgements

7.0   Disclosures

The authors declare no conflict of interest.

8.0   References

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