ORIGINAL REPORT

Group O blood donors in Iran: evaluation of isoagglutinin titers and immunoglobulin G subclasses

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This study aims to determine the most frequent titers of anti-A and anti-B (both presumed immunoglobulin [Ig]M and IgG) in Iranian group O blood donors and to compare these titer values with those found in other studies. In addition, alloantibody production and plasma levels of four IgG subclasses were compared between the high-titer and non–high-titer study groups. This study investigated anti-A and anti-B titers in 358 plasma samples. Based on these results, two study groups (high-titer and non–high-titer) were formed. Antibody detection tests were performed to detect unexpected antibodies to D, C, c, E, e, K, k, Fy^a, Fy^b, Jk^a, Jk^b, M, N, S, s, P1, Le^a, and Le^b. Four IgG subclasses were also evaluated through nephelometry assay. The most frequent titer obtained by room temperature and indirect antiglobulin tube tests was 256. The frequency of titers ≥512 was 31.5 percent. None of the cases showed unexpected RBC alloantibodies. IgG2 levels were significantly higher in the high-titer group. Evaluation of isoagglutinin titers in group O Iranian blood donors can provide insight into the frequency of isoagglutinin titers both within the Iranian population and as compared with other populations. A significant difference in IgG2 levels between the high-titer and non–high-titer groups was identified. More investigation needs to be conducted on the root cause of this finding. ***Immunohematology* 2021;37:5–12.**

Key Words: isoagglutinin, transfusion, antibody titration, blood donor, IgG subclasses

The presence of high levels of anti-A and anti-B in a blood plasma component can cause unfavorable immune responses in recipients. These immune responses may cause severe red blood cell (RBC) destruction that can also be fatal.[1,2] There is an increased risk of hemolytic reactions after an ABO-minor mismatched platelet transfusion.[3,4] The U.S. Food and Drug Administration has also issued reports of death after the transfusion of plasma components with high levels of isohemagglutinins.[5] Despite the fact that some transfusion service providers have a policy against using high-titer plasma components, specifically platelet concentrates, there is still a lack of agreement on the threshold titer value that would lead to an unsafe blood component.[6,7] Diet, as well as ethnic and environmental factors, play a contributing role in the level of isoagglutinin titers in different populations; therefore, it is important to determine the frequency of high isoagglutinin titers in each population and also to determine the cutoff titer value that determines a plasma component to be unsafe.[8,9]

Unexpected alloantibodies are normally produced through immunization by blood transfusion or pregnancy. Alloantibodies such as anti-M, anti-P1, anti-H, and anti-I can occur without previous RBC exposure. It has been shown that microbial elements or environmental factors are also involved in alloantibody production.[10] Alloantibodies are important in transfusion medicine and can cause complications, especially if they are present at high levels.[11] Alloantibodies to RBC antigens have been reported in about 0.8 percent of blood donors.[12]

Generally, antibody formation against RBC antigens depends on many factors, including the nature of the antigen and its immunogenicity, environmental factors, and immune system variations in the individual. Antibodies can be produced at high levels in high responders.[13,14] Immunoglobulin (Ig)G antibodies are typically clinically significant in transfusion medicine, and all four IgG subclasses can be produced in an individual with high-titer isoagglutinins.[15]

In this study, we aimed to find the most frequent titer of anti-A and anti-B (IgM and IgG) in Iranian group O blood donors. Because there are no previous studies on the frequency of isoagglutinin titers in Iran, our study will assess information from group O blood donors in this country for the first time to highlight the importance of studying isoagglutinin titers in each unique population. Furthermore, this study compares alloantibody production and plasma levels of four IgG subclasses between the high-titer and non–high-titer study groups.

Materials and Methods

Sample Collection

Blood samples were obtained from voluntary non-remunerated group O blood donors in the Iranian Blood

Transfusion Organization (IBTO). Each sample was collected in a tube containing EDTA as an anticoagulant. About 10 mL of each blood sample was centrifuged at 200*g* for 5 minutes at 22°C. Then, 4 mL RBC-free plasma was separated, aliquoted, and stored at −30°C until testing. A total of 358 plasma samples from both men and women donors with an age range of 18–68 years were tested. The samples were collected randomly within 11 months between November 2014 and October 2015.

Preparation of A_1 and B RBC Suspensions

To prepare A_1 and B RBCs for use in testing, 2 group A_1, D– packed RBC units and 2 group B, D– packed RBC units were separately pooled. Testing of the pools confirmed their ABO group, a negative antibody detection test, and a negative direct antiglobulin test. The pooled RBCs were washed three or four times until a clear supernatant was reached. A 2–5 percent RBC suspension was prepared with Alsever's solution containing antibiotics. The method for preparing these RBCs was validated with commercial reagent group A_1 and B RBCs from Bio-Rad (Cressier FR, Switzerland).

Tube Test

Twofold serial dilutions in normal saline (0.9%) from 1:2 to 1:1024 were made for each sample. If necessary, higher dilutions were prepared. For each sample, 100 µL diluted plasma was transferred to the next tube in sequence until the final dilution was reached. Two sets of diluted tubes were prepared for each sample: one to determine IgM titer and one to determine IgG titer of anti-A and anti-B.

The room temperature (RT) method was used as a semiquantitative method to determine IgM titer.[16–18] By this method, 100 µL of each dilution was incubated with 50 µL of the 2–5 percent pooled A_1 or B RBCs separately in a tube test for 1 hour at RT. After incubation, samples were centrifuged immediately at 1060*g* for 15 seconds (EBA 21 centrifuge; Hettich, Kirchlengern, Germany). Results were read starting from the tube with the lowest plasma concentration (1:1024). The titer endpoint was the highest dilution with visible agglutination (1+ reactivity) after gentle shaking.

To determine IgG titer, an indirect antiglobulin test (IAT) was performed. By this method, 100 µL diluted sera was incubated with 50 µL of the 2–5 percent pooled A_1 or B RBCs at 37°C for 45 minutes and then washed three times to eliminate unattached antibodies. Two drops of polyspecific antihuman globulin (AHG) (anti-IgG/anti-C3d; Bio-Rad, Feldkirchen, Germany) were added to each tube.[18,19] IgG-coated reagent RBCs (in-house preparation; IBTO, Tehran, Iran) were added to each tube with a negative reaction to validate the washing process. Endpoint titers were determined in the same way as that for the RT method.

Column Agglutination Technology

Because both IgM and IgG antibodies may participate in agglutinating RBCs using RT and IAT methods, samples with initial titers >256 (113 of 358 samples) were evaluated by semi-automated column agglutination technology (CAT).[20]

Treatment of Plasma with DTT

Dithiothreitol (DTT) (0.01 M) was used to inactivate IgM antibody in the plasma samples before testing by CAT. The DTT was prepared by dissolving 0.154 g DTT powder (BDH Chemicals, Poole, UK) in 100 mL phosphate-buffered saline (pH 7.3). The positive control was prepared using monoclonal anti-D (IgM, D-13088 [Sifin, Berlin, Germany] monoclonal test reagent) diluted 1:4 in 2 percent albumin. The negative control was an IgG anti-D (CE-Immunodiagnostica, Eschelbronn, Germany). To inactivate the IgM antibodies, 1 mL of each donor plasma sample was incubated with 1 mL of 0.01 M DTT at 37°C for 1 hour. For the positive and negative controls, 100 µL of each control was treated with 100 µL of the 0.01 M DTT.

Testing by CAT

Twofold serial dilutions of each DTT-treated plasma from 1:2 to 1:1024 were made. Finally, 25 µL of diluted plasma and 50 µL of the A_1 or B RBC suspensions diluted to 0.8 percent (INVITROLISS; MTC Invitro Diagnostics AG, Bensheim, Germany) were added to the low-ionic-strength solution (LISS)/Coombs card (AHG-Coombs MTC; MTC Invitro Diagnostics AG). IAT results were recorded after the cards incubated at 37°C for 15 minutes and then centrifuged at 85*g* for 10 minutes. The endpoint titer was the highest dilution giving visible macroscopic agglutination (1+ reactivity).

Since some of the blood donor samples showed high levels of isoagglutinins, even without previous RBC exposure, it became of interest to investigate alloantibody production and IgG subclasses. In this study, we compared levels of alloantibody and IgG subclasses within two groups of individuals (high-titers vs. non–high-titers). Thus, 22 plasma samples (11 samples with titers greater than 512 [high-titer group] and 11 samples with titers less than 128 [non–high-titer group]) were selected randomly. Unexpected antibodies and IgG subclasses were evaluated on these samples.

Alloantibody Detection

The presence of antibody against D, C, c, E, e, K, k, Fy^a, Fy^b, Jk^a, Jk^b, M, N, S, s, P1, Le^a, and Le^b antigens was evaluated using standard reagent RBCs: Cell I, Cell II, and Cell III. These three reagent RBCs were prepared according to the Immunohematology Reference Laboratory at IBTO.

Three tubes were labeled as I, II, and III for each sample, and two drops of plasma were added to each tube. One drop of reagent RBCs I, II, and III was added to the labeled tubes, respectively. Tubes were centrifuged at 1000*g* for 15–20 seconds, and hemagglutination or hemolysis was examined in each tube. Two drops of LISS were added to each tube as an enhancer. After a 10- to 15-minute incubation at 37°C, the tubes were centrifuged and examined. The results were read and recorded. Then, after washing three times, polyspecific AHG was added to the tubes. After centrifugation, each tube was read for agglutination and the final result immediately recorded. To validate the negative AHG results, one drop of sensitized RBCs (IgG-coated control RBCs) was added to tubes with negative results.[21] (Note: IgG-coated control RBCs were prepared in-house by incubating anti-D reagent [Anti-D incomplete, titer = 128; CE-IMMUNODIAGNOSTIKA, Gmbh, Neckargemund, Germany] diluted to adjust titer, as needed, with 6% albumin [Iranian Blood Research and Fractionation Co., Tehran, Iran]) with D+ RBCs at 37°C for 60 minutes and then washed three times to remove the supernatant.) This control showed ≤2+ results by the IAT, thus validating the negative AHG reactions.

IgG Subclasses

Plasma levels of IgG1, IgG2, IgG3, and IgG4 (mg/L) were measured by nephelometry assay using the Binding Site kit (MININEPH, Birmingham, UK). A total of 40 µL MININEPH Hu reagent plus 400 µL specific buffer were added to the defined amounts of plasma dilution according to the manufacturer's instructions (for IgG1, 10 µL of 1:11 plasma dilution; for the other three subclasses, 20 µL of 1:121 dilution). High- and low-level controls were used in the assays according to the manufacturer's instructions.

Statistical Analysis

All data were analyzed by standard statistical software (SPSS, Chicago, IL). A p value <0.05 was considered statistically significant. To evaluate the distribution of the high-titer isoagglutinins in different age ranges and also by different methods of titration, Kruskal-Wallis and Mann-Whitney tests were used. A comparison between the two groups (high-titer and non–high-titer) was performed in GraphPad (San Diego, CA) Prism version 5.04 using the *t* test (Mann-Whitney).

Results

Tube Test

Among 358 samples, the frequencies of titration endpoints ≥512 in the four measured titers were as follows: IgM anti-A (10.3%), IgM anti-B (5.5%), IgG anti-A (23.4%), and IgG anti-B (12.3%). The frequencies of anti-A titers (IgM and IgG) ≥512 was higher than those of anti-B titers (IgM and IgG) (p = 0.0001). The detailed results are shown in Table 1. Figure 1 depicts the frequency of titer endpoints for IgG (anti-A and anti-B) and IgM (anti-A and anti-B).

Table 1. Frequency of the highest titer of anti-A and anti-B observed with IgM and IgG antibodies in tube testing

	IgM		IgG	
Endpoint titer	Anti-A, *n* (%)	Anti-B, *n* (%)	Anti-A, *n* (%)	Anti-B, *n* (%)
512	27 (7.5)	17 (4.7)	64 (17.9)	37 (10.3)
1024	9 (2.5)	3 (0.8)	17 (4.7)	7 (2.0)
2048	0	0	3 (0.8)	0
4069	1 (0.3)	0	0	0
≥512	37 (10.3)	20 (5.5)	84 (23.4)	44 (12.3)

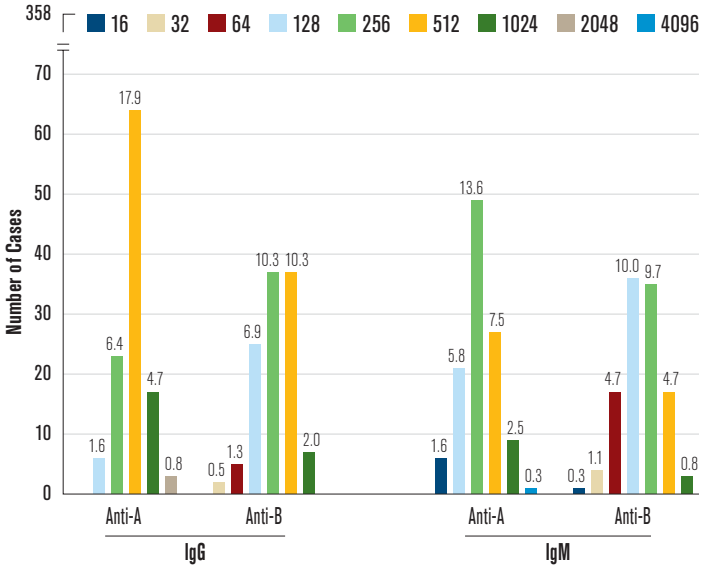

Fig. 1 The frequency (%) of titer endpoints (16–4096) for IgG (anti-A and anti-B) and IgM (anti-A and anti-B) by tube method.

Among 358 group O blood donors, 113 samples showed at least one endpoint titer ≥512 when tested by tube method (RT or IAT), giving a titer frequency of 31.5 percent. (Note: when both IgM and IgG titers were ≥512, they were counted as one case.) A total of 30 samples (8.3%) showed a titer >512. Only three samples (0.8%) had a titer >1024.

Figure 2 shows a comparison between men and women group O blood donors in our study population. Most of the randomly selected donors were men (329, 91.9%); only 29 (8.1%) female donors were included in our study. Although men constituted the majority of donors in this study, the percentage of titers ≥512 was higher among women (48.2% vs. 21.1%). Table 2 compares high-titer values within the male and female populations. Among men, 30 (9.1%) and 70 (21.1%) samples showed a titer ≥512 for IgM anti-A and IgG anti-A by tube method, respectively. For anti-B titers, 17 (5.1%) and 39 (11.8%) of the samples from men had a titer ≥512 for IgM and IgG, respectively. In women, 8 (27.5%) and 14 (48.2%) samples were observed with a titer ≥512 for anti-A IgM and IgG, respectively. Anti-B titers of ≥512 in samples from the women were found at 10.3 percent (n = 3) and 17.2 percent (n = 5) for IgM and IgG, respectively. Considering a p value <0.05 as significant, the comparison shows that high-titer IgM anti-A and IgG anti-A are more prevalent in women (p = 0.002 and 0.001, respectively).

Donors having anti-A or anti-B titers ≥512 (RT or IAT) were divided into five age ranges (Figs. 3 and 4). This analysis showed no significant relationship between age range and the probability of titers ≥512 except for IgM anti-B (RT method). The frequency of titers ≥512 for IgM anti-B is lower in the 39–48 years age range in comparison with other age ranges (p = 0.019).

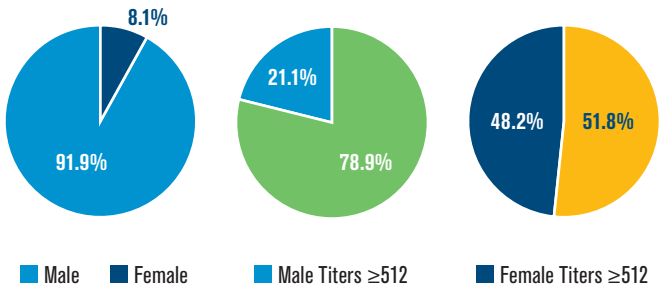

Fig. 2 Comparison of titers by male and female group O blood donors. Titers ≥512 are found in higher percentages in the female donor group.

Table 2. Percentage of titers ≥512 for male and female donors

	Room temperature		Indirect antiglobulin test	
Donors	**Anti-A, % (*n*)**	**Anti-B, % (*n*)**	**Anti-A, % (*n*)**	**Anti-B, % (*n*)**
Men	9.1 (30)	5.1 (17)	21.1 (70)	11.8 (39)
Women	27.5 (8)	10.3 (3)	48.2 (14)	17.2 (5)
Men vs. women				
p	0.002	0.25	0.001	0.4
Odds ratio	3.8	2.1	3.5	1.55
95% CI	1.55–9.3	0.58–7.7	1.59–7.49	0.56–4.3

CI = confidence interval.

CAT-DTT Results

We tested 113 samples having titers ≥512 by CAT after DTT treatment of the plasma (CAT-DTT). The titer after DTT treatment decreased to ≤256 in 85 samples for anti-A and in 91 samples for anti-B. The mean titer decreased by one or two dilutions in CAT-DTT tests. Twenty-eight of 113 (7%) samples had IgG titers for anti-A, and 22 samples (19.4%) had IgG titers for anti-B ≥512 in CAT-DTT tests (18 samples: 512; 9 samples: 1024; and 1 sample: 2048). Because IgG is best active at 37°C, it is better that it be measured without the influence of IgM.[21] Table 3 shows endpoint titers of donors classified by test method (RT, IAT, and CAT-DTT methods).

Alloantibody Detection Results

Results showed no unexpected alloantibody(ies) detected in any of the samples. Considering the fact that high-titer isoagglutinins are present in some blood donors, our investigation showed that there was no relation between the incidence of potentially unsafe group O donors with titers ≥512 and alloantibody production.

Results of IgG Subclasses

Evaluating the levels of four IgG subclasses in high-titer and non–high-titer groups showed a significant difference in IgG2 levels between the two groups. Other IgG subclasses did not display any significant differences. Comparisons are shown in Figure 5 (p < 0.05 was considered significant).

Discussion

Frequency of Anti-A and Anti-B Titers Vary in Different Populations

According to an international forum, high-titer iso-agglutinins are a potential problem in transfusion medicine, and a critical titer should be determined as a cutoff for plasma components when considering transfusion of these

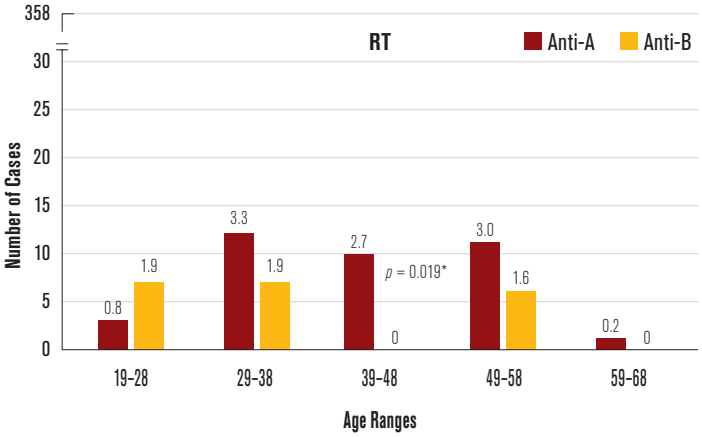

Fig. 3 Frequency (%) of titers ≥512 in different age ranges observed in room temperature (RT) testing by tube method. *IgM anti-B titer is lower in the 39–48 years age range in comparison to other age ranges (p = 0.019).

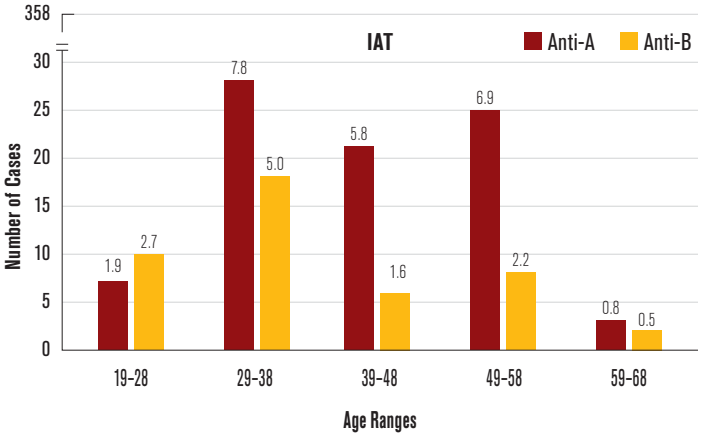

Fig. 4 Frequency (%) of titers ≥512 in different age ranges observed in the indirect antiglobulin test (IAT) by tube method.

components to patients with ABO-incompatible RBCs.[18] Because the titer of isoagglutinins is affected by environmental factors as well as immune system variations in the donor, the frequency of high-titer isoagglutinins could vary in various populations. Therefore, it is essential to determine the prevalence of high-titer cases in each population.[8,22] Testing of anti-A/-B titers in several countries showed variation in testing methods as well as differences in the critical high-titer cutoff value (Table 4).[2,18,22–26]

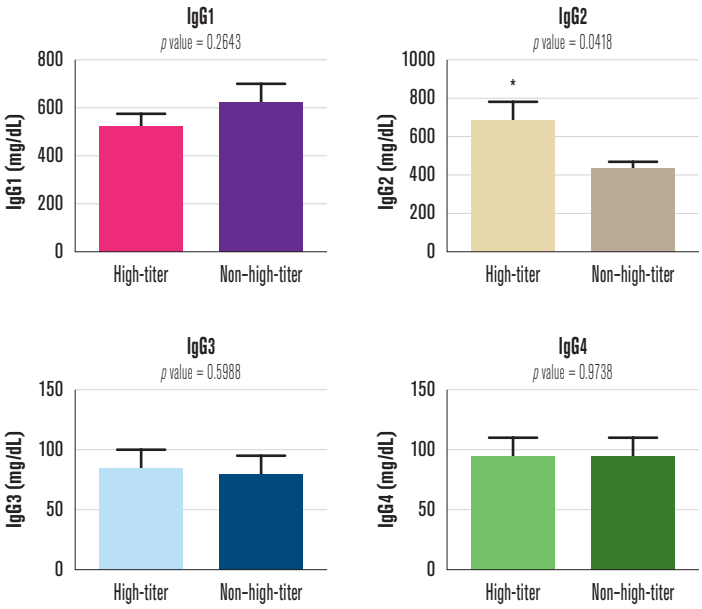

Fig. 5 Comparison of immunoglobulin (Ig)G subclass levels in plasma samples from high-titer and non–high-titer groups. The p value is shown in each evaluation, and $p < 0.05$ was considered significant. The amount of IgG2 subclass showed a significant difference between the two groups.

Table 3. Endpoint titers of donors classified by test method (N = 113)

	Room temperature		Indirect antiglobulin test		CAT-DTT	
Endpoint titer	**Anti-A, % (*n*)**	**Anti-B, % (*n*)**	**Anti-A, % (*n*)**	**Anti-B, % (*n*)**	**Anti-A, % (*n*)**	**Anti-B, % (*n*)**
16	0	0.8 (1)	0	0	0	0
32	0	3.5 (4)	0	1.7 (2)	0	0
64	5.3 (6)	15 (17)	0	4.4 (5)	5.3 (6)	6.1 (7)
128	18.5 (21)	31.8 (36)	5.3 (6)	22.1 (25)	18.5 (21)	28.3 (32)
256	43.3 (49)	30.9 (35)	20.3 (23)	32.7 (37)	51.3 (58)	46 (52)
512	23.8 (27)	15 (17)	56.6 (64)	31.8 (36)	15.9 (18)	12.3 (14)
1024	7.9 (9)	2.6 (3)	15 (17)	6.1 (7)	7.9 (9)	7 (8)
2048	0	0	2.6 (3)	0	0.8 (1)	0
4096	0.8 (1)	0	0	0	0	0

CAT-DTT = column agglutination technology using dithiothreitol-treated plasma.

Several studies have been performed to understand the severity of complications caused by the high level of isoagglutinins through transfusion.[6,27] There is no universal agreement on a critical titer and methodology. Nevertheless, the RT tube method is generally performed, and titer endpoint of 128 has been determined as a critical titer in many countries.[28] In the gel method and other automated systems, titer endpoints differ from those for the tube method.[29] This finding also applied to our study.

In this study, 128 (IgM) and 256 (IgG) are the most frequently observed titers; these titers are considerably higher than critical titers in many countries. Only a few samples were found with a titer <64 or >512. We have shown that the prevalence of titers ≥512 is higher among women. Based on other studies, higher ABO antibody formation in women could be due to more sensitization from pregnancy or infections.[30–32] The isoagglutinin titer is important in transplantation (especially bone marrow transplantation) and early spontaneous abortion as well.[33–36]

Table 4. Critical isoagglutinin titers in different countries using different methods

Country	Method	Critical titer
Brazil	Tube	64
Czech Republic	RT	64
Finland	RT	32
Germany	RT	64
India	Tube	128
Italy	Gel	64
	IAT	256
Japan	IAT	512
Norway	IAT	250
Sweden	RT	100
	IAT	400
UK	Automated	100
	Tube	128
United States	Tube	50
	Gel	200

IAT = indirect antiglobulin test; RT = room temperature using saline in tube. From Nay.[23]

Isoagglutinins that sensitize or agglutinate at 37°C, especially at higher titers, may be the most potentially critical. On the other hand, ABO antibodies reactive at RT probably do not cause any clinical complications. In this study, the frequency of titers >512 was 8.4 percent and the frequency of titers ≥512 was 31.5 percent using the tube method. These findings are comparable with high-titer cases in Brazilian (30%) and Italian (27%) studies.[37,38] Our study was an *in vitro* investigation that, for the first time, showed the most frequently encountered titers of ABO antibodies in Iranian donors and compared titer values in male and female donors as well as within age ranges of these donors. We note that a clinical study is also required to determine potentially clinically critical titers in practice.

Alloantibodies and IgG Subclasses

This study, for the first time, evaluates alloantibodies and IgG subclasses in high-titer and non–high-titer group O blood donors in the Iranian population. It has been shown that different antibody responses against polysaccharides and susceptibility to different bacterial infections are probably under the control of genetic conditions and have a relationship with total IgG2 levels in serum.[39,40] In high responders, IgG2, IgG3, and IgG4 are produced at higher levels in comparison with low responders, who have different allotype heavy chains.[41] Immunoglobulin allotypes are related to high and low antibody responses, and a high responder against a particular antigen may also be a high responder to many other antigens. This statement is also true about low responders.[39] In our study, the plasma level of IgG2 was significantly higher in the high-titer group. We did not find any correlation between high isoagglutinin production and unexpected RBC alloantibody production. It is of note that while most of the non-ABO RBC antigens to which clinically significant alloantibodies are formed are proteins or glycoproteins, ABO antigens are carbohydrates. Naturally occurring anti-T, directed at a carbohydrate antigen, may be a better antibody for comparison.

In conclusion, our study shows that the most frequent titer of ABO antibodies in Iran is 256, and the frequency of titers ≥512 is 31.5 percent in group O blood donors using the tube method. Additionally, no connection between alloantibody and isoagglutinin production was observed in this study. IgG2 levels were found significantly higher in donors with high levels of isoagglutinins.

Acknowledgments

SA collected data, analyzed the data, performed the tests, and wrote the manuscript. MMog revised the manuscript, collected data, and cooperated with amendments. AAP designed the study. AA and MMos revised the tables and

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