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Immunoglobulin Class Profiles of ABO Antibodies in Saliva and Serum of Healthy Individuals

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ABO · Saliva · Antibodies · Flow cytometry · Infections

Abstract
Introduction: The coronavirus disease (COVID-19) pandemic gave rise to studies investigating the association of ABO blood group with COVID-19 susceptibility. It is hypothesized that ABO antibodies might play a role in neutralizing SARS-CoV-2. However, ABO antibodies were exclusively analyzed in blood samples. Investigation of ABO antibodies in saliva, an easy-to-obtain surrogate for respiratory secretions, may provide novel insights into mucosal immunity crucial in early defense against respiratory pathogens. ***Methods:*** In this study, saliva and serum samples from healthy individuals with known blood groups were investigated using a flow cytometric method for separate anti-A/anti-B IgA, IgM, and IgG class antibody detection. Saliva samples were additionally tested using hemagglutination-based neutral and indirect anti-human globulin test gel cards. This method comparison was complemented by dilution experiments with a high-titer anti-A/anti-B WHO standard. ***Results:*** In saliva, IgA was the most abundant ABO antibody class, followed by IgM; IgG was detected only in low levels in all non-AB blood types. In serum, IgM was the predominant ABO antibody class in all non-AB blood types, followed by IgA and IgG, the latter mainly detected in group O individuals. Saliva and serum samples of group O individuals yielded the highest variability of ABO-specific antibody levels. Regardless of sample material and blood type, major interindividual differences in ABO antibody reactivities were recorded. Antibody levels correlated moderately between these two body fluids. There were no significant sex and age-group differences in ABO antibody levels in both serum and saliva. WHO standard dilution experiments yielded technique-specific limits of detection, illustrating the inherent differences of immunofluorescence versus agglutination. ***Conclusion:*** For the first time, salivary ABO antibodies were investigated by separate detection of the three most relevant antibody classes IgA, IgM, and IgG in a healthy cohort. This study opens new perspectives regarding mucosal ABO antibody class profiles and their potential influence on respiratory infections.

Introduction

The coronavirus disease (COVID-19) pandemic gave rise to studies investigating the association between COVID-19 susceptibility and ABO blood groups [1–3]. Specifically, among infected individuals, blood group O was underrepresented compared to non-group O-types. It was hypothesized that natural ABO antibodies may be a main factor of protection, particularly anti-A of the IgG class, as this was detected preferably in samples of group O individuals [4, 5].

So far, ABO antibodies were exclusively analyzed in blood samples. However, mucosal immunity, particularly secretory IgA, is reported to play a more important role in early defense against respiratory pathogens [6]. Saliva

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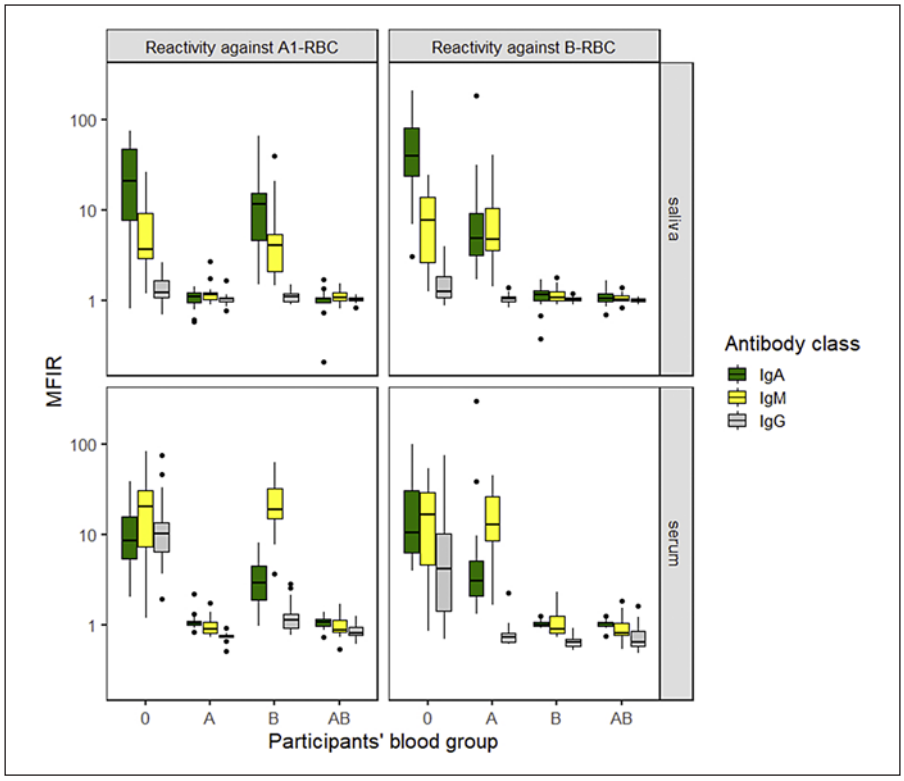

Fig. 1. Boxplots representing MFIR of flow cytometric ABO antibody detection in saliva and serum. Values on the y-axis are displayed on a log(10) scale.

may represent an easy-to-obtain surrogate for respiratory secretions and was insufficiently studied regarding ABO antibodies [7–9]. No data are available on salivary ABO antibodies regarding their immunoglobulin (Ig) classes and corresponding serum levels. Reasons might be challenges in saliva processing caused by high proteolytic activity, viscosity, and composition [10].

In this study, a flow cytometric method was established to differentially detect IgA, IgM, and IgG ABO antibodies in saliva. ABO antibody reactivities in the saliva of healthy individuals were put in relation to serum levels. Aside from providing novel insights into ABO-dependent physiological human salivary composition, this method can be applied to investigate the dynamics of salivary ABO antibody levels in pathological conditions such as COVID-19 [11].

Materials and Methods

Whole blood and saliva samples were collected from apparently healthy Austrian donors with known ABO blood group. Saliva was donated spontaneously after abstaining from food, liquid, or chewing gum for at least 1 h. Saliva specimens were immediately centrifuged (10 min with 15,000 *g* at 4°C) to remove cells and debris. Sera and salivary supernatants were frozen at −20°C until analysis.

Flow cytometric analysis of ABO antibodies in saliva was prepared by incubating 50 µL of saliva containing 0.5 µL protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO, USA) with 50 µL of 0.05% of red blood cells (RBCs; A1, B, and O, diluted in PBS buffer, all serologically typed C-D-E–, K-). After incubation at 20°C for 30 min and washing, 25 µL of saturating secondary antibodies (Alexa Fluor™ 488 $F(ab')_2$ goat anti-human IgA α chain-specific 1:100, R-PE fab fragment goat anti-human IgM $Fc_{5\mu}$-specific 1:50, Alexa Fluor™ 647 fab fragment goat anti-human IgG Fc_{γ}-specific 1:50; Jackson Immuno Research, West Grove, PA, USA) were added. After incubation (30 min at 4°C), washing, and resuspension, RBCs were aspirated twice through a 25-gauge needle to disaggregate potential RBC agglutinates. Detection of ABO antibodies in serum was prepared by the incubation of 25 µL of serum and 25 µL of 0.1% RBCs (previously treated with Karnovsky's Fixative) [12] under otherwise the same conditions as saliva samples. Flow cytometry was performed on a FACS Canto II using FACS Diva software (BD Biosciences, Heidelberg, Germany). Single RBCs were gated by FSC-A versus FSC-H. Median fluorescence intensities (MFIs) were measured in the FITC, PE, and APC channels. Per batch, one group O and one group AB sample served as positive and negative controls, respectively. MFI ratios (MFIR) were calculated by MFI sample of interest/MFI of an AB-sample. Based on the MFIR of all samples, the cut-off for positive results was set at the highest MFIR for each antibody class obtained with O RBCs (online Suppl. Table 1; for all online suppl. material, see www.karger.com/doi/10.1159/000527233).

Serum and saliva samples (the latter equally pretreated with protease inhibitor cocktail, as mentioned above) were additionally tested using hemagglutination-based neutral (NaCl) and indirect anti-human globulin test (IAT) gel cards (MicroTyping system, Bio-Rad, Vienna, Austria) at 20°C and 37°C, respectively, according to manufacturer instructions for serum testing. Results were expressed as agglutination scores (negative, borderline +/−, 1+ to 4+).

Performance of flow cytometry was assessed using a high-titer anti-A/anti-B WHO reference reagent (National Institute for Biological Standards and Control, Hertfordshire, England, UK) in a 1-in-2 dilution series (diluted with AB-serum and AB-saliva pools

Table 1. Number of saliva and serum samples according to their ABO-specific reactivities by flow cytometry

Sample material	Participant's blood group	Samples with ABO type-congruent reactivity in any Ig class (%)	Samples with ABO type-incongruent reactivity in any Ig class (%)[a]	Ig class	anti-A		anti-B		Total
					neg (%)	pos (%)	neg (%)	pos (%)	
Saliva	0	21 (100) anti-A or -B	0 (0)	IgA	1 (5)	20 (95)	0 (0)	21 (100)	21
				IgM	5 (24)	16 (76)	6 (29)	15 (71)	
				IgG	10 (48)	11 (52)	8 (38)	13 (62)	
	A	25 (100) anti-B	1 (4) anti-A	IgA	25 (100)	0 (0)	0 (0)	25 (100)	25
				IgM	25 (100)	0 (0)	4 (16)	21 (84)	
				IgG	24 (96)	1 (4)	22 (88)	3 (12)	
	B	19 (100) anti-A	2 (10) anti-B	IgA	1 (5)	18 (95)	18 (95)	1 (5)	19
				IgM	8 (42)	11 (58)	19 (100)	0 (0)	
				IgG	14 (74)	5 (26)	18 (95)	1 (5)	
	AB	13 (93) negative	1 (7) anti-A/-B	IgA	13 (93)	1 (7)	13 (93)	1 (7)	14
				IgM	14 (100)	0 (0)	14 (100)	0 (0)	
				IgG	14 (100)	0 (0)	14 (100)	0 (0)	
Serum	0	21 (100) anti-A or -B	0 (0)	IgA	2 (10)	19 (90)	0 (0)	21 (100)	21
				IgM	3 (14)	18 (86)	3 (14)	18 (86)	
				IgG	0 (0)	21 (100)	6 (29)	15 (71)	
	A	24 (100) anti-B	0 (0) anti-A	IgA	24 (100)	0 (0)	7 (29)	17 (71)	24
				IgM	24 (100)	0 (0)	2 (8)	22 (92)	
				IgG	24 (100)	0 (0)	23 (96)	1 (4)	
	B	17 (100) anti-A	0 (0) anti-B	IgA	7 (41)	10 (59)	17 (100)	0 (0)	17
				IgM	0 (0)	17 (100)	17 (100)	0 (0)	
				IgG	14 (82)	3 (18)	17 (100)	0 (0)	
	AB	16 (0) negative	0 (0)	IgA	16 (100)	0 (0)	16 (100)	0 (0)	16
				IgM	16 (100)	0 (0)	16 (100)	0 (0)	
				IgG	16 (100)	0 (0)	16 (100)	0 (0)	

[a] All four saliva samples with incongruent results were negative in the respective routine gel card assays.

as well as PBS; 5 replicates each). For these experiments, only saliva from non-secretors was employed to ensure the absence of soluble blood group substances; non-secretor status was ascertained using the Wiener agglutination inhibition test [13]. Precision, dynamic range, and limit of detection were defined as described elsewhere [14, 15]. Data management and statistical analyses were performed using R Version 4.1.0.

Results

Samples were obtained from 88 participants (44 females and 44 males; group O, $n = 21$; A, $n = 25$; B, $n = 19$; AB, $n = 23$). The median age was 46 years, ranging between 19 and 69 years. Blood for serum testing was not available from one A and two B participants. Of 23 AB participants, 7 donated both saliva and serum; further, 7 and 9 donated only saliva and serum, respectively.

Results of flow cytometry are presented in Figure 1; Table 1, as well as online Supplementary Figure 1. All 65 non-AB-saliva samples had detectable ABO antibodies of any Ig class. The predominant ABO antibody class was IgA, detected in all O-, A-, and 18/19 (95%) B-saliva samples, followed by IgM, which was consistently detectable in O-, A-, and in a lower proportion in B-saliva samples. In comparison, the variability of IgG in saliva was very limited in all blood types (see online Suppl. Table 2). Of 62 non-AB sera, 61 (98%) showed positive reactions in any Ig class. All antibody classes were clearly detected in the sera of group O participants. IgA and IgM were consistently detected in A and B sera, with a higher proportion of positive IgM versus IgA (group A/anti-B: 22/24, 92% positive IgM vs. 17/24, 71% positive IgA; group B/anti-A: 17/17, 100% positive IgM vs. 10/17, 59% positive IgA). The proportions of positive IgM versus IgA differed significantly in group B (anti-A, $p = 0.007$) but not in group A (anti-B, $p = 0.137$). IgG was rarely observed in A and B sera. All AB-serum and 13/14 AB-saliva samples showed no anti-ABO reactivity. Samples of group O individuals yielded the highest variability of ABO-specific antibody levels.

The Spearman correlation revealed an association between IgA ABO antibodies in the saliva and serum of A and B donors ($\rho = 0.76$, $p < 0.001$ for anti-B in group A; $\rho = 0.56$, $p = 0.02$ for anti-A in group B, respectively, see online Suppl. Table 3). There was a significant association between IgM anti-B ($\rho = 0.51$, $p = 0.019$) and IgG anti-A

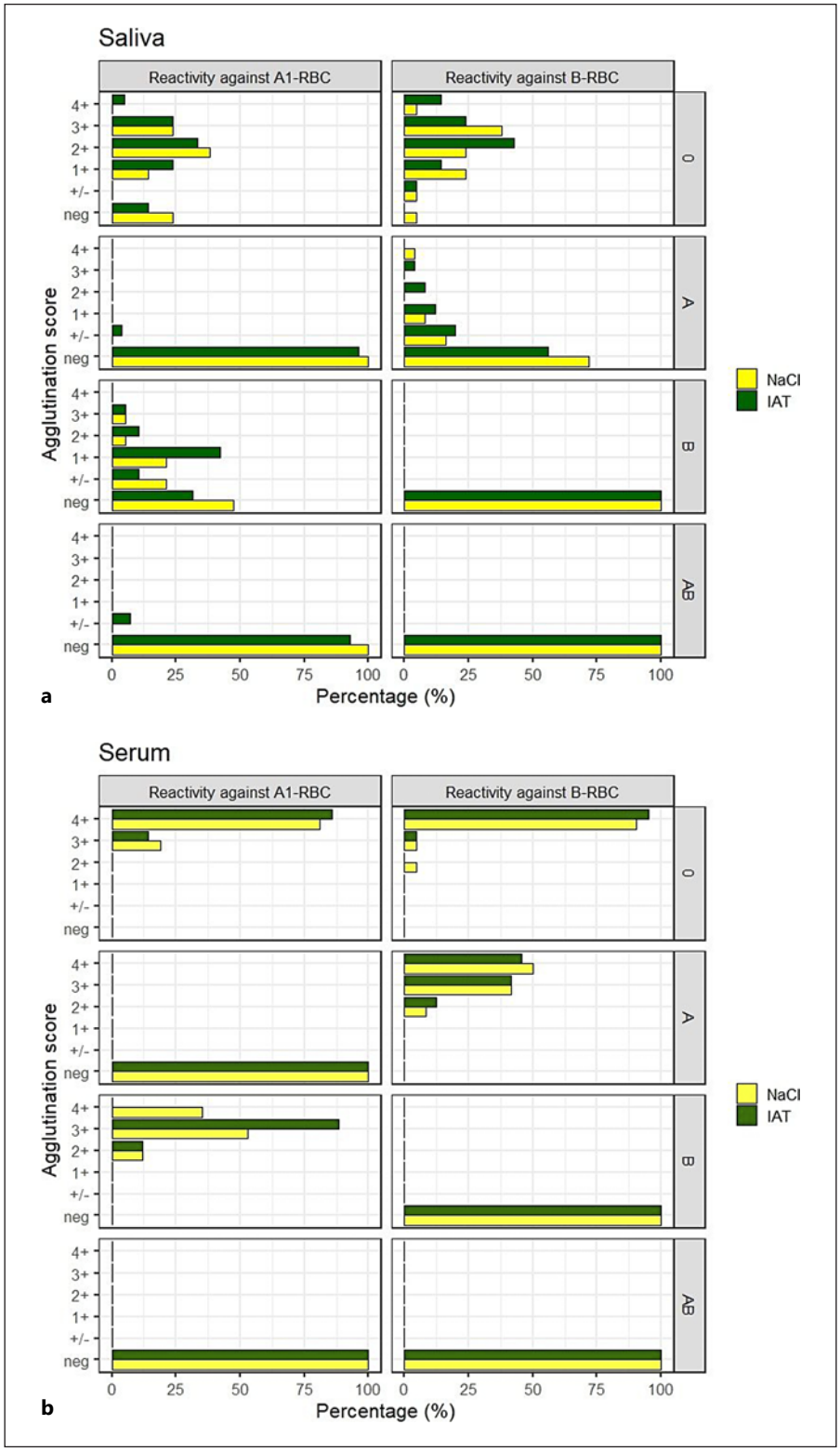

Fig. 2. a ABO-specific reactivities of saliva samples determined by gel cards. One A- and one AB-saliva sample showed borderline reactivities with A1 RBCs, most probably due to mucin clots. Both were negative in flow cytometry. **b** ABO-specific reactivities of serum samples determined by gel cards.

($\rho = 0.62$, $p = 0.003$) in the saliva and serum of O donors. There were no significant sex and age-group differences in anti-A/anti-B MFIRs in both serum and saliva.

Saliva and serum test results in gel cards (NaCl and IAT) are shown in Figure 2a, b and online Supplementary Table 4. In brief, all group O-saliva samples were reactive with A and/or B RBCs, whereas only 6/19 (32%; NaCl) and 11/19 (58%; IAT) group B-saliva samples had detectable anti-A. Positivity rates for group A were even lower (anti-B in NaCl: 3/25, 12%; IAT: 6/25, 24%). No saliva sample reacted with O RBCs, whereas one A and one AB saliva appeared borderline reactive with A RBCs,

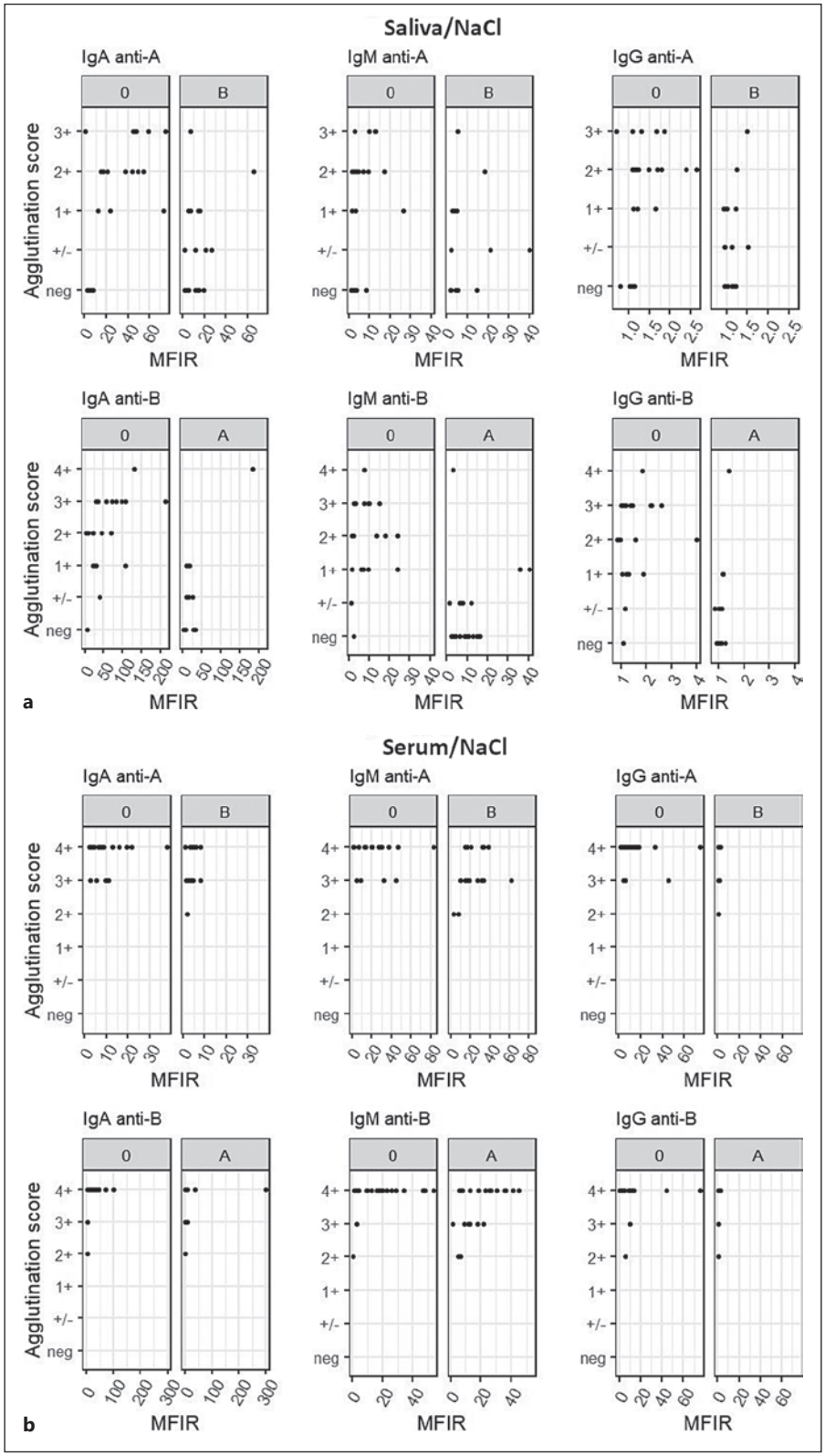

Fig. 3. a Relation between results of NaCl gel cards (y-axis) and anti-A and anti-B MFIR in IgA, IgM, and IgG (x-axis) in saliva. Plots are displayed separately by relevant blood types. **b** Relation between results of NaCl gel cards (y-axis) and anti-A and anti-B MFIR in IgA, IgM, and IgG (x-axis) in serum. Plots are displayed separately by relevant blood types.

most probably due to mucin clots. All sera (100%) reacted according to their blood group in both NaCl and IAT, with the majority of non-AB sera being 4+ or 3+ reactive. The agglutination scores were not consistently associated with MFIR of flow cytometry, reflecting the fact that agglutination was based on combined ABO reactivity of different Ig classes (see Fig. 3, 4a, b).

The high-titer anti-A/anti-B WHO reference reagent showed strong IgM (MFIR anti-A = 95.4, anti-B = 92.1) and moderate IgG levels (MFIR anti-A = 20.3, anti-B =

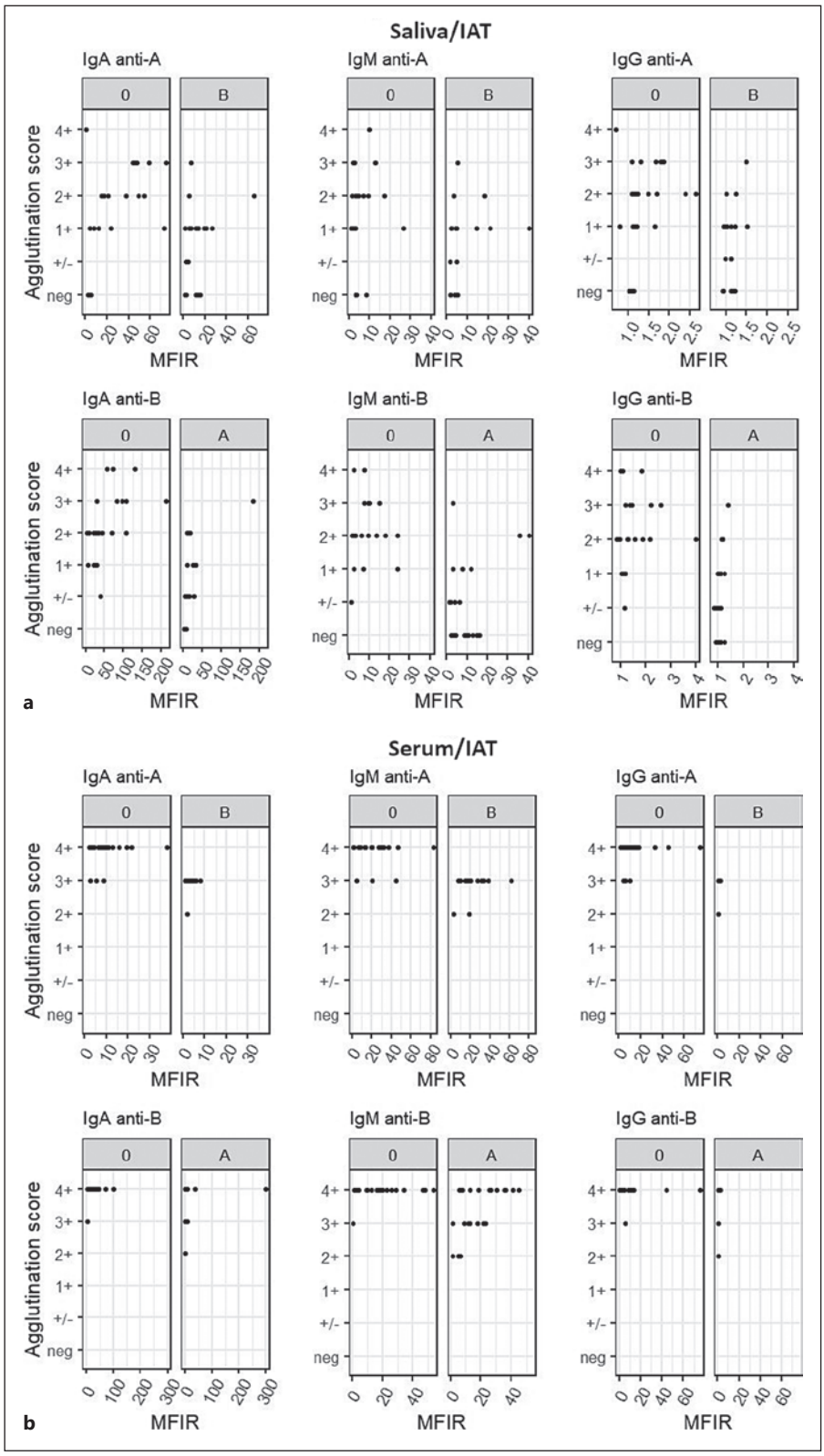

Fig. 4. a Relation between results of IAT gel cards (y-axis) and anti-A and anti-B MFIR in IgA, IgM, and IgG (x-axis) in saliva. Plots are displayed separately by relevant blood types. **b** Relation between results of IAT gel cards (y-axis) and anti-A and anti-B MFIR in IgA, IgM, and IgG (x-axis) in serum. Plots are displayed separately by relevant blood types.

8.4) in flow cytometry. In addition, considerable amounts of IgA (MFIR anti-A = 15.4, anti-B = 41.7) were also detected. Titration experiments using three different diluents (AB-serum and -saliva pools, as well as PBS) revealed that the AB-serum pool had an inhibitory effect on anti-A and -B reactivities in both flow cytometry and hemagglutination as compared to the AB-saliva pool and PBS. Precision defined as relative standard deviation (% RSD) over the 5 replicates and averaged across dilution steps varied between 1.6 and

Table 2. Flow cytometric performance assessment determined by dilution of high titer anti-A/anti-B WHO standard with three different diluents in 5 replicates

Diluent	Ig class	A1 RBC					B RBC				
		flow cytometry				gel card titer NaCl/IAT	flow cytometry				gel card titer NaCl/IAT
		precision (mean % RSD)	DR	LOB	LOD (titer)		precision (mean % RSD)	DR	LOB	LOD (titer)	
AB-Serum Pool	IgA	2.8	15.4	94.9	111.5 (16)	16/128	2.2	41.7	111.1	114.3 (64)	16/128
	IgM	7.4	95.4	93.6	128.7 (64)		4.6	92.1	89.8	92.0 (128)	
	IgG	8.9	20.3	56.4	66.9 (64)		5.2	8.4	103.3	106.9 (2)	
PBS	IgA	2.8	23.4	71.8	74.0 (256)	128/256	2.1	59.0	86.6	90.4 (512)	128/256
	IgM	7.9	144.8	63.8	70.5 (512)		5.5	133.8	63.0	69.5 (512)	
	IgG	6.5	100.9	22.2	24.0 (512)		4.1	44.0	27.3	29.6 (512)	
AB-Saliva Pool	IgA	1.6	15.4	93.3	94.7 (256)	64/256	1.9	41.6	112.3	114.3 (128)	32/128
	IgM	3.8	113.8	60.6	62.5 (1,024)		3.6	108.0	63.3	64.8 (1,024)	
	IgG	6.1	69.0	18.5	20.0 (1,024)		5.4	29.4	28.1	29.0 (512)	

Parameters were defined according to Armbruster 2008 [13] and Wood 2013 [14]. RSD, relative standard deviation; DR, dynamic range (ratio between largest (non-diluted)/smallest (blank) fluorescent signal); LOB, limit of blank (Mean blank + 1.645*SD); LOD, limit of detection (=LOB + 1.645*SD highest dilution).

8.9%. The broadest and narrowest dynamic ranges were obtained by dilution with PBS (up to ratio 144.8 for A1-specific IgM) and AB-serum pool, respectively (Table 2). Regression analyses (online Suppl. Fig. 2AB) revealed a better linear fit (R^2 = 0.89–1.0) for A1 RBCs than for B RBCs (R^2 = 0.66–1.0).

Using gel cards, the WHO reference reagent showed anti-A/anti-B reactivity according to the manufacturer's instructions when diluted with PBS (1:128 in NaCl, 1:256 in IAT). Diluted with the AB-serum pool, anti-A/anti-B titers were 1:16 and 1:128 in NaCl and IAT, respectively. Dilution in AB-saliva pool revealed an anti-A titer of 1:64 and 1:256 and an anti-B titer of 1:32 and 1:128 in NaCl and IAT (Table 2).

Discussion

In this study, a flow cytometric method for separate IgA, IgM, and IgG class-specific ABO antibody detection in saliva was devised. Investigation of a cohort of healthy individuals revealed that IgA was the most abundant ABO antibody class in saliva, followed by IgM and IgG. Total IgA serves as major immunological defense on mucous membranes and is produced locally, similar to IgM. In contrast, as total salivary IgG is mostly serum-derived, it should mirror serum levels, which, however, was not the case for ABO antibodies [6]. All group O and only few A and B individuals had ABO-specific IgG antibodies in serum. In saliva, comparatively fewer group O samples than group B and A samples demonstrated IgG (76%, 26%, and 12%, respectively).

In serum, IgM was the most abundant ABO antibody class, followed by IgA and IgG, the latter mainly detected in group O individuals. Surprisingly, IgA was detected in similar levels to IgG in group O and predominantly to IgG in group A and B individuals. Hence, the traditional division of ABO antibodies into IgM and IgG should be reconsidered in light of the significant presence of IgA. Regardless of sample material, major interindividual differences in ABO antibody reactivities were recorded. With the applied cut-off level strategy, serum testing yielded no unspecific/false-positive reactions at all, and only few unexpected reactivities in saliva. Using O RBCs for the cut-off definition seemed adequate as they do not carry ABO antigens and the respective antibodies were not expected to bind. This cut-off definition leads to a low-level threshold of positivity and should therefore be interpreted with caution.

Saliva represents a poorly studied sample material for ABO antibody detection, as all previous studies were hemagglutination-based [7–9, 16]. Compared to serum, saliva is a heterogenic and unsterile body fluid influenced by several factors like viscosity, composition (water, enzymes, mucins, and antibodies), and microbiome [10]. Standardized sampling circumstances are essential to maintain the integrity of potential biomarkers [17]. A major challenge in saliva processing is its high proteolytic activity that has been overcome by use the of protease inhibitors [10]. Previous experiments had shown that

protease inhibitors exerted only minimal reductions of MFIR (<10%) and no effect on hemagglutination scores in gel cards. Moreover, varying mucin concentrations in saliva specimens may increase the possibility of evaluation errors. ABO antibody levels in saliva might be prone to volatility dependent on fluid balance. Serum levels of ABO antibodies remain stable over time in individuals but demonstrate a large heterogeneity within donor populations [18–20].

Comparison of flow cytometry with the standard hemagglutination technique reflected by gel card testing was additionally performed by dilution experiments using a high-titer anti-A/anti-B WHO reference reagent that has been previously used by Sprogøe et al. [21] to benchmark flow cytometric anti-A IgM and IgG measurements. In that study, flow cytometric results were reported as absolute entities (molecules of equivalent fluorochrome) by relating sample MFI to a standard curve obtained by calibration beads. This methodological setup is quite different from ours, making direct comparison difficult. Our method enables calculating MFIR based on AB-samples serving as negative controls, allowing for evaluating the relative antibody content. This approach has proven efficient for judging different clinical conditions, such as the influence of infections on ABO antibodies or ABO-incompatible transfusion and transplantation [11, 12, 19, 20].

WHO reference reagent titration experiments using three different diluents (AB-serum and -saliva pools as well as PBS) revealed that AB sera had an inhibitory effect on anti-A and anti-B reactivities in both flow cytometry and hemagglutination. We assume that this inhibition was caused by soluble A and B substances in AB sera, which competitively bind anti-A and anti-B of the WHO standard. These blood group substances are common in the serum of both secretors and non-secretors and in other body fluids of secretors [22, 23]. Therefore, care was taken to select exclusively AB-saliva samples of non-secretors for pooling.

Standard serological techniques are based on RBC agglutination and fail to clearly discriminate between Ig classes. It is a common misconception that direct agglutination in neutral gel cards is solely brought about by IgM. In fact, the combined action of different Ig classes (not only IgM but also IgG as well as IgA) will, in concert, lead to direct agglutination. The same holds true for IAT (Coombs card milieu) where not only anti-human globulin-driven agglutination (IgG) but also direct agglutination via IgM and IgA can be observed. For these reasons, a direct comparison of Ig class-specific flow cytometric MFIR and mixed Ig class hemagglutination titers may be misleading (see Fig. 3, 4a, b).

Regarding test performance, flow cytometry supersedes standard semiquantitative hemagglutination techniques by measuring all cell-bound ABO antibodies regardless of their agglutination potential. Preferably, IgM-induced RBC agglutination is prevented by the use of fixed RBCs for serum testing and needle aspiration prior to analysis, both commonly used to obtain single RBCs for flow cytometry without affecting antibody binding [12, 19, 24–26]. Moreover, the use of Ig class-specific secondary antibodies allows for differentiation of ABO antibodies into IgA, IgM, and IgG, including IgG subclasses [19, 20]. Numerical values enable objective and quantitative results. Despite its high-throughput ability, flow cytometry needs more processing time (due to several incubation and washing steps, instrument setting, analysis, and evaluation) compared to standard hemagglutination techniques, which additionally can also be performed and evaluated manually. Both techniques feature comparable costs for reagents and consumables.

Another possibility to quantitatively measure ABO antibodies is by ELISA, which works with the use of synthetic or animal ABO glycans coated onto a microplate surface [27–29]. Anti-A/anti-B antibodies in sera bind these glycans and are detected by enzyme-labeled anti-human IgM or IgG. ELISA techniques are less elaborate than flow cytometry but exhibit an incomplete antigenic structure due to the non-human origin of ABO glycans and the potential conformation loss during the coating procedure. As complete human ABO on intact RBCs could not ideally be reflected by this assay, test sensitivity and specificity has shown to be decreased in comparison to flow cytometry and standard hemagglutination techniques [19].

Detection of salivary ABO antibodies provides previously unknown aspects of mucosal immunity that are possibly important in the current COVID-19 pandemic [11]. So far, no data were available on salivary ABO antibody classes or the association of these antibodies between saliva and serum. Further studies are required to determine the potential mutual influence of infections and mucosal ABO antibody class profiles.

Statement of Ethics

This study was approved by the Ethics Committee of the Medical University of Vienna (EK Nr. 1248/2021). All study participants gave written informed consent.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Funding Sources

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Author Contributions

Marlies Schönbacher established and performed the analyses and wrote the manuscript. Chiara Banfi and Andrea Berghold calculated the statistics. Eva Matzhold and Thomas Wagner discussed the results and contributed to the final version of the manuscript. Wolfgang Mayr provided helpful advice in interpreting the data. Günther F. Körmöczi designed and supervised the study and wrote the manuscript. All the authors critically revised the manuscript.

Data Availability Statement

Further data are available by request after approval granted by the Ethics Committee of the Medical University of Vienna.

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