ORIGINAL REPORT

Effect of long-term frozen storage on antibodies in blood samples: immunoglobulin, tetanus toxin antibody, and ABO antibody

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The Japanese Red Cross Blood Services archives donor blood samples from all donations for look-back surveys. These samples, approximately 5 million per year, are stored frozen at –30°C for 11 years and are subsequently discarded. Because such samples may also be valuable for antibody-related research and other purposes after the mandated storage period, we evaluated their potential applicability, with a particular focus on the preservation of antibody levels. Fresh samples were compared with frozen-stored samples (–30°C for 14 years: 11 years of mandatory good manufacturing practice storage period + 3 years until measurement) for immunoglobulin levels (IgM, IgG, IgA, and IgE) as a general indicator of humoral immunity, tetanus toxin antibody titers as a long-lived vaccine-induced antibody, and ABO antibody titers (saline-direct agglutination test and saline-indirect antiglobulin test [saline-IAT]), which are factors in blood group typing and hemolytic transfusion reactions. For immunoglobulin levels, comparisons of non-paired fresh and frozen-stored samples were made from a random donor population (IgM, IgG, IgA: 105 cases each, IgE: 32 cases). For tetanus toxin antibody and ABO antibody, comparisons were conducted from the same donors with fresh and frozen-stored samples (tetanus toxin antibody: 47 cases, ABO antibody: 12 cases). No significant differences were observed in immunoglobulin levels, tetanus toxin antibody titers, or ABO antibody titers measured by the saline-direct agglutination method. Saline-IAT–measured ABO antibody titers were significantly lower in frozen-stored samples. Detailed results were as follows: (1) Median immunoglobulin levels (fresh vs. frozen-stored): IgM, 78.5 mg/dL versus 86.7 mg/dL; IgG, 1240.2 mg/dL versus 1280.2 mg/dL; IgA, 241.0 mg/dL versus 245.3 mg/dL; and IgE, 0.24 µg/mL versus 0.12 µg/mL. (2) Tetanus toxin antibody titers—geometric mean titer (GMT) (95% confidence interval [CI]): fresh 0.23 (0.12–0.43) IU/mL versus frozen-stored 0.28 (0.17–0.46) IU/mL. (3) ABO antibody titers—GMT (95% CI): saline-direct agglutination, fresh 65.4 (33.3–128.5) versus frozen-stored 51.7 (24.9–107.4); saline-IAT, fresh 391.0 (160.3–953.6) versus frozen-stored 248.7 (107.8–573.6). The evaluated immunoglobulin levels were largely preserved, suggesting that archived blood samples retain sufficient stability for antibody research. However, careful study design is necessary to minimize potential sample deterioration.

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Key Words: storage, immunoglobulin, tetanus toxin antibody, anti-A and anti-B, freezing

Frozen-stored blood samples are invaluable resources for serial cross-sequential and longitudinal studies, enabling the investigation of temporal trends in immunologic and hematologic parameters.[1,2] The Japanese Red Cross Blood Services (JRCBS) archives a sample from every blood donation, storing it frozen for look-back surveys as part of their hemovigilance system, in compliance with good manufacturing practice (GMP) standards.[3–6] Beyond their primary hemovigilance purpose, these archived samples have recently been leveraged effectively in epidemiologic research in Japan after the mandatory storage period had elapsed.[7–9]

Although previous studies have examined the effects of freezing/thawing[10–17] and short-term frozen storage[11,15,18] on immunoglobulin levels, there is limited evidence regarding the impact of prolonged storage exceeding 10 years.[19] This limitation represents a critical knowledge gap for research applications that rely on long-term preserved samples.

The present study addresses this gap by systematically evaluating the effects of long-term frozen storage (–30°C, 14 years) on human immunoglobulin levels, vaccine-induced antibodies, and ABO blood group antibodies. Specifically, we measured (1) immunoglobulin levels (IgM, IgG, IgA, and IgE) as general indicators of humoral immunity, (2) tetanus toxin antibody titers as representative of long-lived vaccine-induced antibodies,[20–23] and (3) ABO antibody titers (saline-direct agglutination test and saline-indirect antiglobulin test [saline-IAT]), which are critical for the prevention of hemolytic transfusion reactions when ABO-incompatible plasma must be transfused, such as in the setting of human leukocyte antigen (HLA)-matched platelet transfusions where ABO-incompatible components may need to be selected to prioritize HLA compatibility.[2,24–27]

Importantly, control samples were selected to account for antibody-specific characteristics (including potential sex differences or age-related declines in antibody titers[28]) as well as the presence or absence of pre-storage data from the same donors enabling paired analyses. Unfortunately, paired analyses were not feasible in some cases because of the unavailability of pre-storage data. By combining a relatively large number of samples with careful methodologic design, this study provides novel and robust evidence regarding the stability of long-term frozen blood samples, supporting their potential utility in future immunologic, transfusion, and epidemiologic research.

Materials and Methods

Samples and Donor Data

Frozen-stored serum samples (–30°C) were collected from blood donations in 2010 and preserved in accordance with GMP requirements for the look-back survey.[3–6] After the mandatory storage period had expired, samples scheduled for disposal were selected for inclusion in this study. These archived samples remained frozen at –30°C until measurement, which was performed in 2024. Before testing, samples were thawed at room temperature (RT).

Control samples comprised fresh serum obtained as residual material after routine testing at the Kanto-Koshinetsu Block Blood Center (Tokyo, Japan). Donor information, including age, sex, and ABO blood group, was retrieved from the JRCBS donor management system. In Japan, eligible blood donors range in age from 16 to 69 years, and the donor population is predominantly male (approximately 71% male vs. 29% female).[29] Detailed procedures for each comparison are described below and in Table 1.

Comparison of Immunoglobulin (IgM, IgG, IgA, and IgE) Levels Between Fresh and Frozen-Stored Samples

To evaluate the effects of long-term frozen storage on immunoglobulin levels, serum samples were obtained from age- and sex-stratified, non-identical donors from 2024 (fresh group) and 2010 (frozen-stored group). For IgM, IgG, and IgA, 105 samples were analyzed per group, and for IgE, 32 samples were analyzed per group (Table 1). To minimize confounding effects from freeze-thawing, fresh samples were subjected to a single freeze-thaw cycle before measurement. Immunoglobulin concentrations were measured using enzyme-linked immunosorbent assay (ELISA) kits (IgM 88-50620, IgG 88-50550, IgA 88-50600, IgE 88-50610; Thermo Fisher Scientific, Tokyo, Japan), according to the manufacturer's instructions. In brief, ELISA plates were coated with pre-titrated purified anti-human monoclonal capture antibody in phosphate-buffered saline (PBS) and blocked with 0.1 percent Tween 20 and 1 percent bovine serum albumin (BSA) in PBS. Diluted serum and two-fold serial dilutions of standards with 0.05 percent Tween 20 and 0.5 percent BSA in PBS were dispensed into each well and incubated at RT for 2 hours. Pre-titrated, horseradish peroxidase (HRP)-conjugated antihuman monoclonal antibody was then added to each well and incubated at RT for 1 hour. Tetramethylbenzidine (TMB) substrates were added to each well and incubated at RT for 15 minutes in the dark. The absorbance of each specimen at 450 nm was measured, and antibody concentrations were calculated.

Comparison of Tetanus Toxin IgG Titers Between Fresh and Frozen-Stored Samples

As a representative antigen-specific antibody, vaccination-derived antibodies against tetanus toxin were evaluated. Most Japanese birth cohorts have been routinely immunized against tetanus, and these antibody titers are generally well preserved with aging.[30] Therefore, paired comparisons between fresh and frozen-stored samples from the same donors were

Table 1. Design of comparison according to each antibody

Antibody evaluated	*n* (donors)	Donor	Sampling	Test
Immunoglobulin types	IgM, IgG, IgA: 105; IgE: 32	Random	Fresh: 2024 Frozen-stored: 2010	Simultaneously (2024)
Tetanus toxin antibody	47	Identical	Fresh: 2024 Frozen-stored: 2010	Simultaneously (2024)
ABO antibody	8 (12 cases)	Identical	Fresh: 2010–2012 Frozen-stored: 2010	At different times (fresh: 2010–2012, frozen-stored: 2024)

possible, providing a reliable assessment of the effects of long-term storage.

Paired serum samples were obtained from donors whose original samples had been frozen in 2010 and who donated again in 2024 (n = 47 per group) (Table 1). Donors were selected from birth cohorts between 1970 and 1990, following the initiation of universal tetanus immunization in Japan in 1968.[22,31] Fresh samples were subjected to a single freeze-thaw cycle before measurement. Tetanus toxin IgG titers were measured using ELISA (EIA-3514R; DRG Instruments, Marburg, Germany), according to the manufacturer's instructions. Microtiter wells, used as a solid phase, were coated with tetanus toxoid antigen. After washing, diluted samples and controls were pipetted into these wells and incubated for 1 hour at 37°C. HRP-conjugated antihuman IgG antibodies were dispensed into the wells and incubated for 30 minutes at RT. After a second washing step, TMB substrates were added to each well and incubated for 15 minutes at RT in the dark. Optical density at 450 nm was measured, and antibody levels were calculated. Geometric mean titers (GMTs) were calculated after $\log_{10}$ transformation.[32]

Comparison of ABO Antibody Titers Between Fresh and Frozen-Stored Samples

Serum samples were evaluated for ABO antibody titers using a two-fold serial dilution method by the conventional tube test, including both the saline-direct agglutination test after incubation at RT for 15 minutes and the saline-IAT after incubation at 37°C for 30 minutes.[33] The detailed selection criteria for the study samples are described below. At the Japanese Red Cross Kanto–Koshinetsu Block Blood Center, when ABO-incompatible platelet components are supplied, ABO antibody titers are routinely measured to assess incompatibility with recipients (anti-A, anti-B, or both).[34,35] Between 2010 and 2012, 4950 blood components were tested for ABO antibody titers using saline-direct agglutination testing (1905 group O and 3045 non-group O components). Subsequently, all group O components were further tested using the saline-IAT, whereas non-group O components were tested by IAT only when the direct agglutination titer was ≥128 (IAT: 1905 group O and 470 non-group O components).

For the present study, donors with available initial saline-IAT titers were selected. Among these donors, only eight group O donors made repeat donations during a defined period in 2024 and were therefore enrolled. The number of cases exceeded the number of individual donors (Table 1; 12 cases from eight donors) because both anti-A and anti-B were measured in some group O donors. For these selected cases, corresponding frozen-stored samples were retrieved. ABO antibody titers from frozen-stored samples were measured and compared with those titers obtained from fresh samples. Frozen-stored titers were measured blindly by two independent investigators using in-house prepared A and B reagent red blood cells (contracted product between the JRCBS, Tokyo, Japan, and FUJIFILM Wako Pure Chemical Corp., Osaka, Japan). Titers were expressed as GMTs, calculated from $\log_2$-transformed values.

Statistical Analysis

Descriptive statistics were reported as median values with interquartile ranges (IQRs) for each immunoglobulin level and as GMT with 95 percent confidence interval (CI) for tetanus toxin antibody and ABO antibody titers. The GMT is suitable for skewed serologic data sets and is robust in representing central tendency.[32,36,37] The GMT is the average of data measured on a logarithmic scale. To calculate GMT, each reported value was log-transformed. Then the logarithms were summed and divided by the number of observations. Finally, the antilogarithm of the average logarithm was calculated to obtain the GMT. Antibody levels in the test groups (frozen-stored) were compared with the corresponding control groups (fresh) using paired t tests or Mann-Whitney U tests as appropriate. When we performed paired t tests, the log-transformed values were tested.[38] Pearson correlation coefficients were calculated between fresh and frozen-stored tetanus toxin antibody values and ABO antibody values. Statistical comparisons between a donor's characteristics of fresh and frozen-stored samples, when not from the same individual, were performed using independent two-sample t tests for age and by χ^2 test for sex. A p value <0.05 was considered statistically significant. All statistical analyses were conducted using software (R Software, version 4.5.1; R Foundation for Statistical Computing, Vienna, Austria).

Results

Comparison of Immunoglobulin (IgM, IgG, IgA, and IgE) Levels Between Fresh and Frozen-Stored Samples (Non-Identical Donors)

Age- and sex-stratified serum samples from non-identical blood donors were analyzed to compare immunoglobulin concentrations measured by ELISA in fresh and frozen-stored groups (Table 1; IgM, IgG, IgA: n = 105 per group; IgE: n = 32 per group). There were no statistical differences in the age and sex ratio of donors between fresh and frozen-stored samples (Table 2). No statistically significant differences were observed between fresh and frozen-stored samples for any of the immunoglobulin levels (Mann-Whitney *U* test) (Table 2 and Fig. 1).

Comparison of Tetanus Toxin IgG Between Fresh and Frozen-Stored Samples (Identical Donors)

Tetanus toxin antibody was selected as a representative specific antibody. In Japan, tetanus vaccination has been administered routinely since 1968, with a coverage rate exceeding 99 percent.[30] These antibodies are also prevalent worldwide and represent an important focus of epidemiologic research.[20,21,23] Notably, tetanus toxin antibody titers decline more slowly with age compared with other vaccine-induced antibodies.[30] To assess the effects of long-term frozen storage, paired samples from the same donors were analyzed: serum frozen stored for 14 years (frozen-stored) versus freshly collected samples (fresh) (Table 1). Donors had a median (IQR) age of 33 (28–37) years at the time of frozen-stored sampling and 47 (42–51) years at the time of fresh sampling ($n = 47$; male = 39, female = 8). A high correlation was observed between fresh and frozen-stored samples (Fig. 2; $r = 0.79$, $p < 0.01$). The GMTs (95% CI) were 0.23 (0.12–0.43) and 0.28 (0.17–

Table 2. Comparison of immunoglobulin (IgM, IgG, IgA, and IgE) levels between fresh and frozen-stored samples

Sample parameters	Fresh	Frozen-stored	*p*
IgM, IgG, IgA			
n	105	105	
Age (years)	39 (20–50)	38 (24–51)	NS (0.76)
Sex (male/female)*	87/18 (82.9/17.1)	88/17 (83.8/16.2)	NS (0.85)
IgM (mg/dL)	78.5 (61.4–108.7)	86.7 (63.8–103.2)	NS (0.58)
IgG (mg/dL)	1240.2 (1075.3–1424.8)	1280.2 (1142.9–1371.7)	NS (0.85)
IgA (mg/dL)	241.0 (199.7–288.5)	245.3 (208.6–286.5)	NS (0.71)
IgE			
n	32	32	
Age (years)	44 (38–47)	43 (35–46)	NS (0.26)
Sex (male/female)*	27/5 (84.4/15.6)	26/6 (81.2/18.8)	NS (0.74)
IgE (µg/mL)	0.24 (0.08–0.35)	0.12 (0.10–0.50)	NS (0.84)

*Sex was expressed as the number of cases with the rate (%).
Continuous variables were expressed as median (interquartile range).
NS = not significant.

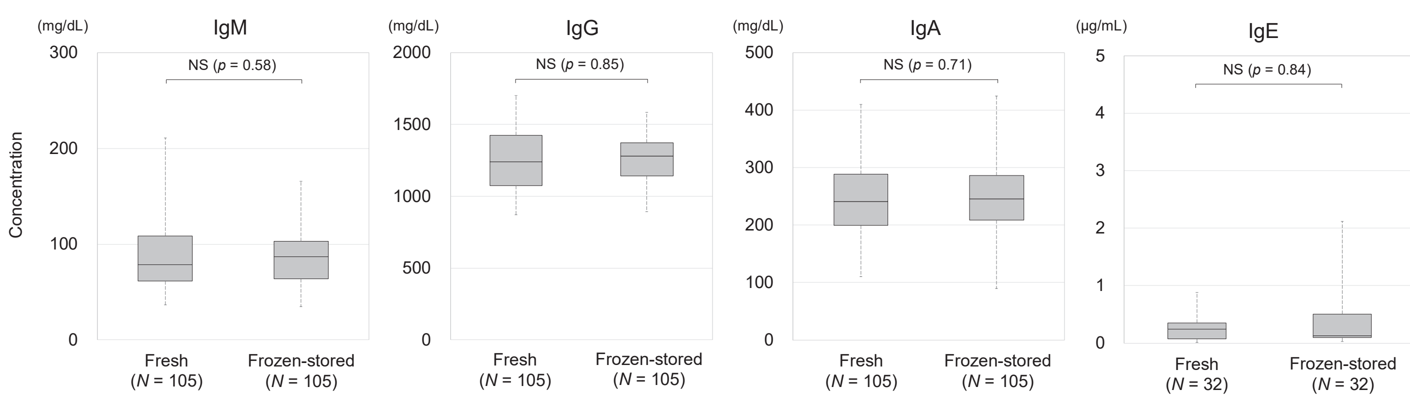

Fig. 1 Comparison of immunoglobulin levels by type (IgM, IgG, IgA, and IgE) between fresh and frozen-stored samples (age-equivalent random populations). Box plots represent 25th to 75th percentile with median (a line in the box) between 2.5th and 97.5th percentile values. Mann-Whitney *U* tests were performed. IgM, IgG, IgA (mg/dL): $n = 105$; IgE (µg/mL): $n = 32$ in each group, respectively. NS = not significant.

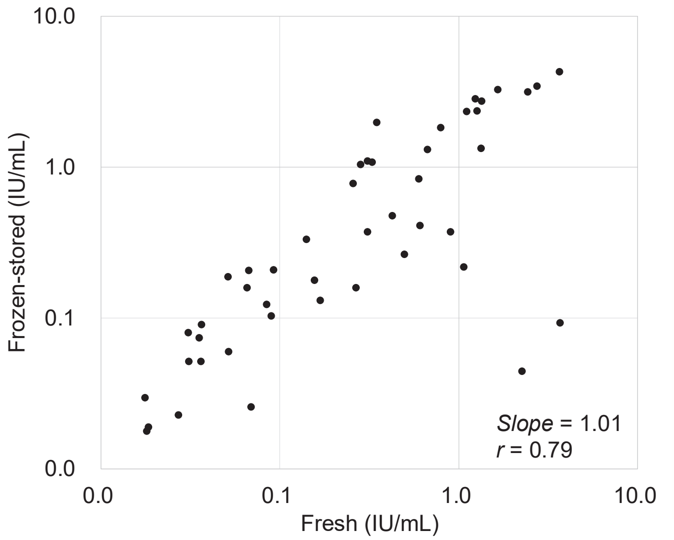

Fig. 2 Correlation of tetanus toxin IgG titers between fresh and frozen-stored samples (identical donors). Scatter plots of tetanus toxin IgG titers are shown. Pearson correlation analysis was performed. $n = 47$.

0.46) in the fresh and frozen-stored groups, respectively. No statistically significant differences were found between fresh and frozen-stored samples (paired *t* test, $p = 0.53$).

Comparison of ABO Antibody Titers Between Fresh and Frozen-Stored Samples (Identical Donors)

The JRCBS routinely measured ABO antibody titers when platelets were supplied as ABO-incompatible components, especially as HLA-matched platelet concentrates. In this study, titers obtained before storage (fresh) were compared with those measured after long-term frozen storage (14 years, frozen-stored) in the same repeat blood donors ($n = 8$; male = 8) (Table 1). The median (IQR) age of donors was 40.0 (35.0–44.5) years. A strong correlation was observed between fresh and frozen-stored titers in both the saline-direct agglutination test and the saline-IAT (Fig. 3; saline-direct agglutination: $r = 0.94$, $p < 0.01$; saline-IAT: $r = 0.96$, $p < 0.01$). The GMTs (95% CI) were as follows: saline-direct agglutination test fresh 65.4 (33.3–128.5) versus frozen-stored 51.7 (24.9–107.4); saline-IAT fresh 391.0 (160.3–953.6) versus frozen-stored 248.7 (107.8–573.6). No statistically significant differences were found in saline-direct agglutination titers (paired *t* test, $p = 0.06$). However, saline-IAT titers were significantly lower in frozen-stored samples compared with fresh (paired *t* test, $p < 0.01$).

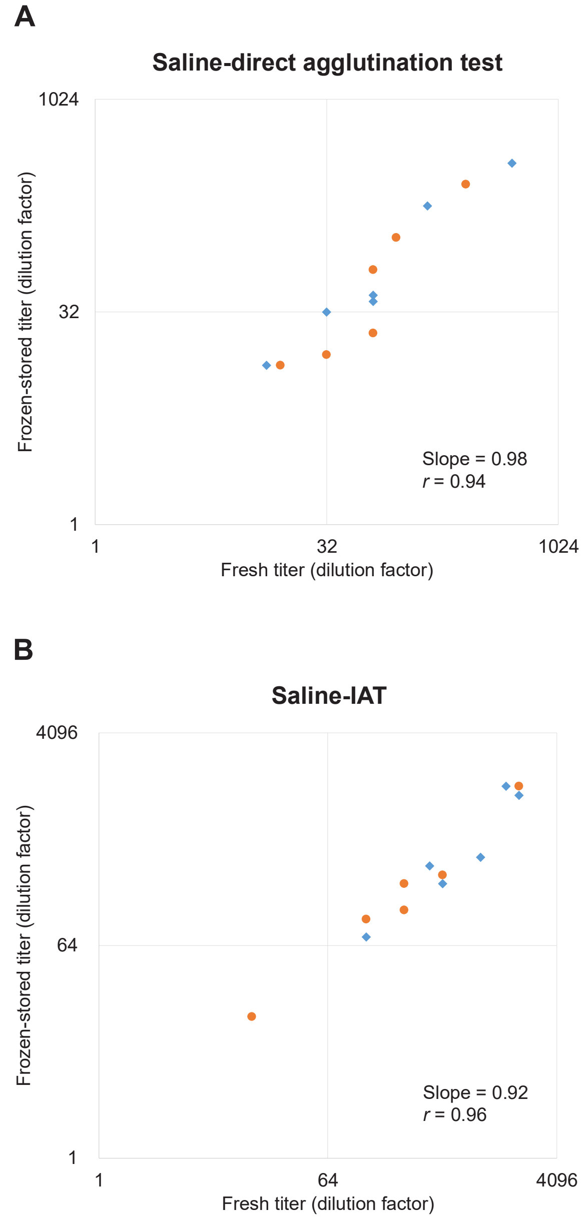

Fig. 3 Correlation of anti-A and anti-B (ABO antibody) titers between fresh and frozen-stored samples (identical donors). Scatter plots of ABO antibody titers were obtained by the conventional tube test in fresh versus frozen-stored samples. Pearson correlation analysis was performed. Saline-direct agglutination test (A) and saline-indirect antiglobulin test (IAT) (B) (12 cases, respectively) values are shown: anti-A, 6 cases (blood group O = 6); anti-B, 6 cases (blood group O = 6); diamond = anti-A; circle = anti-B.

Discussion

In this study, we assessed the effects of long-term frozen storage on blood samples and confirmed that these samples retained adequate antibody levels for research on immunoglobulins, tetanus toxin antibody, and saline-direct agglutination ABO antibodies. While previous studies have reported the effects of freezing-thawing[10,13–17] and short-term storage (1–4 years on anti-*Candida,*[18] 2–3 years on anti-severe acute respiratory syndrome coronavirus type 2 [SARS-CoV-2][15]), few studies have evaluated the effects of long-term storage on antibodies (e.g., 20 years for lyophilized enterovirus equine antisera),[19] highlighting the need for further validation of long-term frozen sample quality.

By leveraging the National Blood Center, we were able to obtain a relatively large number of samples, including paired serum/data from the same donors, which allowed a reliable evaluation of frozen storage effects on antibody levels with high interindividual variability. Comparison methods were designed according to antibody type (Table 1) and were justified as follows: (1) immunoglobulin levels were assumed to be comparable between 2010 and 2024; (2) tetanus toxin titers, which decline minimally with age, allowed paired comparisons over 14 years; and (3) ABO antibody titers were measured before and after storage, representing an ideal design for evaluating frozen-storage effects.

Although long-term frozen storage largely preserved immunoglobulin levels, tetanus toxin antibody and saline-direct agglutination ABO antibody titers, saline-IAT–measured ABO antibody titers were significantly lower in frozen-stored samples. In ABO antibody titers, titers from fresh samples reflect measurements without freezing, whereas the titers from frozen-stored samples reflect the effects of one freeze-thaw cycle. Therefore, long-term frozen storage and freeze-thaw effects may have contributed to the observed differences. Additionally, storage effects may vary depending on the assay; stepwise dilution methods report the final positive dilution as the titer, and long-term storage may have a greater impact on samples with low-antibody concentrations. Because the saline-IAT is highly sensitive due to the addition of antihuman globulin, small storage-related changes may be more apparent in IAT measurements. These findings suggest that careful experimental design is critical when detecting low signals with stepwise dilution methods, particularly when comparing frozen samples with fresh controls. Although a statistically significant decrease was observed in the saline-IAT titers of frozen-stored samples, the GMT ratio remained within a two-fold range, and the IAT-measured ABO antibody titers in frozen samples are suitable for studies where the differences between experimental and control groups are sufficiently large to exceed several-fold. Additionally, when observing changes in antibody levels over time, especially in the same individuals and using the same design adopted in this study for tetanus toxin antibody, the study design should be configured to test hypotheses in which the difference between the experimental and control groups is sufficiently large (e.g., a two-fold change), taking into account not only the changes in antibody levels themselves but also the potential deterioration of the frozen samples.

This study has several limitations. First, the sample size for ABO antibody titer analysis was very small, due to the limited availability of frozen samples with corresponding ABO antibody measurements at the time of study entry, as detailed in the sample selection process in Materials and Methods (Table 1). Second, the study subjects were exclusively male, reflecting the higher proportion of male donors in Japan (approximately a 7:3 male-to-female ratio). Also, the analyzed donors had relatively high ABO antibody titers because of the routine testing criteria. Moreover, with the aging of the Japanese population, most repeat donors are now in their 40s or older. Although our previous study demonstrated an age-related decline in ABO antibody titers, all donors included in the present analysis exhibited relatively high titers. Because ABO antibody titers are influenced by multiple factors, including age, sex, and birth cohort,[39] further studies involving a broader range of donor characteristics are warranted. In addition, whether the biological function of antibodies is fully preserved during long-term frozen storage remains to be determined. Finally, although the storage temperature in this study was −30°C,[10–15,17–19] which is consistent with many previously published reports, biological samples in general biobanks are typically stored at −80°C or lower. Therefore, the findings of this study cannot be directly extrapolated to samples stored at −80°C or below.

Based on these results, frozen archived samples can be further used, especially in epidemiologic studies. Rare human sera are also sometimes used as reference reagents in blood group testing, and there are cases in which long-term frozen serum of unknown specificity yielded new findings after more than 10 years.[40] Therefore, the present study provides valuable reference data for the storage and use of serum in medical laboratories.

Conclusion

While careful consideration must be given to the selection of appropriate research designs and measurement methods, the frozen-stored samples collected through the hemovigilance system for all 5 million annual donations at the

JRCBS, currently discarded after 11 years of storage at –30°C, would constitute a highly valuable resource if made publicly available. Such samples could support not only epidemiologic investigations but also a wide range of related scientific and public health applications.

Ethics Approval

This study was approved by the institutional review board of JRCBS (approval number 2020-032) and conducted in accordance with the principles of the Declaration of Helsinki. Blood donors who donated in 2010 were provided the opportunity to opt out of participation in this study.

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