The Proportion of Long-term Response to Anti-N IgG Antibody after 12 Months for COVID-19 Subclinical Infections and a Longitudinal Survey for COVID-19 Subclinical Infections in 2021

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Abstract:
Objective To examine the continuation of antibody prevalence status after 12 months and background factors in antibody-positive subjects following asymptomatic infection with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2).
Methods We initially determined the SARS-CoV-2 anti-nucleocapsid protein immunoglobulin G (anti-N IgG) antibody prevalence in 1,603 patients, doctors, and nurses at 65 medical institutions in Kanagawa Prefecture, Japan. We then obtained consent from 33 of the 39 subjects who tested positive and performed follow-up for 12 months.
Results Follow-up for up to 12 months showed that a long-term response of the anti-N IgG antibody could be detected in 6 of the 33 participants (18.2%). The proportions with hypertension, using an angiotensin-receptor blocker, and without a drinking habit were higher among the participants with a long-term anti-N IgG antibody response for up to 12 months than among those without a long-term antibody response.
Conclusions The proportion of individuals with subclinical COVID-19 who continuously had a positive result for the anti-N IgG antibody at 12 months was low.

Key words: SARS-CoV-2 Anti-N IgG antibody, immunochromatography, COVID-19, subclinical infection, epidemiological survey

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Introduction

The numbers of infections and deaths worldwide from December 2019 to November 15, 2021, associated with the coronavirus-induced disease 2019 (COVID-19) pandemic induced by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) are 251 million and 5 million, respectively (1). Although we have not yet been able to return to our pre-outbreak way of life, the surrounding environment is being changed with the development of vaccines and implementation of vaccination for COVID-19.

SARS-CoV-2 uses its spike proteins to enter the host by binding to angiotensin-converting enzyme (ACE) 2 receptors present on the surface of cells (2). Researchers all over the world are targeting the spike protein for the development of potential vaccines (3). Therefore, the anti-spike protein (anti-S) antibody, anti-spike protein receptor binding domain (anti-S-RBD) antibody, and neutralizing antibody (NAb) testing cannot be assessed to determine if vaccinated individuals acquired innate immunity prior to vaccination in order to produce antibodies continuously. However, anti-
nucleocapsid protein (anti-N) antibody testing can detect antibodies that have been continuously produced in individuals with a history of infection, with or without vaccination targeting the spike protein (3). With such backgrounds, the number of articles reporting long-term antibody responses after natural infection with the novel coronavirus is limited, and in particular, no long-term follow-up has been reported for long-term antibody response after subclinical infection.

We previously investigated subclinical COVID-19 infections from May to June 2020 (4) and reported the results of up to six months’ follow-up after anti-N immunoglobulin G (IgG) antibody detection, indicating that the proportion of participants with a long-term anti-N IgG antibody response was 24.2% (8 out of 33 individuals) (5). In the present study, further follow-up was conducted afterward to determine the long-term anti-N IgG antibody response after one year. Since there are a certain number of people who cannot be vaccinated or have not been vaccinated, it is clinically meaningful to clarify the status of the long-term anti-N IgG antibody response.

Proportions of individuals with subclinical COVID-19 in various countries were reported in 2020, but the number of reports of subclinical infections is limited in 2021. Data on those infected patients have been reported by various countries and local governments, including the World Health Organization (WHO). Therefore, it is of epidemiologic interest to examine the changes in the proportions of individuals with subclinical infection over time. Our survey conducted in 2020 showed that 39 of 1,603 individuals (2.4%) had subclinical COVID-19 (positive proportion of anti-N protein IgG antibodies). Limited to physicians and nurses, 10 of 504 individuals (2.0%) had a subclinical infection (4).

In the present study, we also report the results of a report on subclinical COVID-19 in 2021 that was concurrently conducted with follow-up for the long-term antibody response mentioned above in the same population as in the 2020 survey, limited to physicians and nurses.

**Materials and Methods**

**Ethics approval and consent to participate**

This study was registered with the Clinical Trials Registry (https://www.umin.ac.jp/; UMIN000040333; May 8, 2020) and was performed in accordance with the study protocol, the Declaration of Helsinki, and the Ethical Guidelines for Clinical Studies of the Ministry of Health, Labour and Welfare of Japan.

This study was approved by the Ethics Review Board of the Kanagawa Physicians Association. All participants provided their written informed consent before participation.

**Study design**

a) Follow-up for up to one year after antibody detection

This multi-center epidemiologic study was conducted at 65 sites in Kanagawa Prefecture. Participants were enrolled from May 18 to June 24, 2020, and followed until July 2021.

The inclusion criteria are as indicated in the previous report (4). Subjects who had a positive result for the SARS-CoV-2 anti-N IgG antibody during the first survey and provided consent for follow-up were followed for the present study.

b) Subclinical COVID-19 survey in 2021

The survey was conducted at 53 sites that agreed to cooperate with the 2021 survey among participant institutions in the 2020 survey indicated in the above survey. Physicians and nurses who participated in the 2020 survey and met the inclusion criterion and none of the exclusion criteria described below were included in the survey. The survey period was from May to July 2021 (reasons for the selection of participants are indicated in parentheses below).

The additional inclusion criterion was provision of consent for the 2021 survey (from an ethical perspective), and the additional exclusion criteria were as follows: a history of novel coronavirus infection (to survey subclinical infections); presence of symptoms including pyrexia, headache, and cough within 21 days after the testing date (to prevent infection during testing); cold symptoms or pyrexia (≥37.5°C) for at least 4 days or severe malaise or labored breathing (because of potential infection) in 2020 or 2021.

Symptoms such as pyrexia due to vaccination were ruled out as exclusion criteria.

**Method**

a) Follow-up for one year after antibody detection

The participants who received a written explanation of the study in order to provide their written consent underwent blood sampling for the first antibody testing after responding to the questionnaire. For participants who had a positive result for the antibody at the initial testing, an explanation of the follow-up was provided in order to obtain their consent in writing, followed by antibody testing at 12 months after antibody detection in order to record clinical symptoms, if applicable.

b) Subclinical COVID-19 survey after one year in 2021

The participants who received a written explanation of the study in order to provide their consent in writing underwent blood sampling for antibody testing after responding to a questionnaire in 2021. The 2021 questionnaire was conducted in order to confirm the participants’ vaccination status as well as whether or not they met the inclusion criterion and none of the exclusion criteria.

**Assay kit**

Cica Immuno-test SARS-CoV-2 IgG was used (6). This is a reagent developed through collaborative research by Professor Akihide Ryo of the Department of Microbiology, Yonago City University Graduate School of Medicine and Kanto Chemical, which detects human SARS-CoV-2 anti-N IgG antibodies contained in the serum of individuals in-
fected with the novel coronavirus. The reagent is based on the principles of immunochromatography. The test was performed immediately after blood collection. When serum containing human SARS-CoV-2 anti-N IgG antibodies is dropped on the sample pad of the test device, human SARS-CoV-2 anti-N IgG antibodies move on the test strip in the test device and form complexes with goat anti-human IgG antibodies labelled with black particles (subsequently “black particle-labelled antibodies”) contained in the conjugate pad. These complexes move to the membrane as they are and bind to viral proteins fixed on the place where a test line will appear. This accumulates black particle-labelled antibodies, which form the test line. If human SARS-CoV-2 anti-N IgG antibodies are not present in the serum, black particle-labelled antibodies are not accumulated, and no test line appears. Whether human SARS-CoV-2 anti-N IgG antibodies are present or not, black particle-labelled antibodies bind to rabbit anti-goat IgG antibodies fixed on the place where a control line will appear. This accumulates black particle-labelled antibodies, and the control line appears. The control line is an indicator of the normal development of the serum. This assay kit was verified by the National Institute of Health Sciences on a simultaneous performance evaluation test of an antibody assay kit against the new coronavirus, and the maximum dilution factor for which all cases were positive was 64-fold (7). The accuracy of this assay kit was equal to or better than that of many other assay kits (7).

Survey items

a) Follow-up for up to one year after SARS-CoV-2 anti-N IgG antibody detection

 Relevant items, such as age and sex, were obtained by the first survey (4), along with clinical symptoms at 12 months after antibody detection and the SARS-CoV-2 anti-N IgG antibody response.

b) Subclinical COVID-19 survey in 2021

SARS-CoV-2 anti-N IgG antibody and vaccination status.

Endpoints

a) Follow-up for up to one year after SARS-CoV-2 anti-N IgG antibody detection

The proportion of individuals positive for the SARS-CoV-2 anti-N IgG antibody was evaluated 12 months after antibody detection. In addition, those participants were divided into two groups based on the presence or absence of a long-term antibody response at 12 months in order to compare participant characteristics based on the response presence. This was followed by an exploratory analysis for factors associated with the long-term antibody response.

b) Subclinical COVID-19 survey in 2021

The proportions of individuals positive for the SARS-CoV-2 anti-N IgG antibody were evaluated among the participants in the 2021 survey.

Statistical analyses

The R software program, version 3.5.2 (R Foundation for Statistical Computing, Vienna, Austria [https://www.R-project.org/]), was used for data analyses. To compare the participant characteristics, Fisher’s exact test and Student’s t-test were used for nominal and continuous variables, respectively. Data from participants who provided unreadable or incomplete questionnaires were excluded from the analysis (only applicable items). The level of significance was 5% for 2-sided tests, and continuous and nominal variables were presented as the mean±standard deviation and number (%), respectively. Wald’s chi-squared test was used for the multivariate analysis among factorial analyses of the long-term antibody response.

Results

a) Follow-up for up to one year after SARS-CoV-2 anti-N IgG antibody detection

The participant flow is indicated in Fig. 1. Among 39 individuals positive for the anti-N IgG antibody during the first subclinical COVID-19 survey in 2020, 33 who provided their consent to participate in the present study underwent follow-up. At 12 months (time points of testing in the applicable participants: Days 355 to 402 after antibody detection), 6 of the 33 participants (18.2%) had a long-term anti-N IgG antibody response. Among the participants with a long-term anti-N IgG antibody response, a cough, runny nose, sputum, and diarrhea were observed during the follow-up period. However, no headache or fever was noted, and none were diagnosed with COVID-19.

The characteristics of participants with or without a long-term anti-N IgG antibody response at 12 months after antibody detection are shown in Table 1. Significant differences were observed regarding the body mass index (BMI), drinking habits, hypertension, and use of angiotensin-receptor blockers (ARBs) between the two groups (Table 1). A multivariate analysis for factors associated with a long-term anti-N IgG antibody response was performed using the age, sex, BMI, and ARB usage as explanatory variables (Supplementary material 1). The age and sex were used as basic characteristics. The BMI and ARB usage were significant variables in the univariate analysis. The result showed that the use of ARB was a significant factor (since proportions of non-drinkers and individuals with hypertension were both 100% in the long-term anti-N IgG antibody response group, those two variables were ruled out).

Since the initial survey was conducted in May and June 2020, none of the patients were vaccinated. Considering the burden on the subjects, if subjects had a negative antibody response during the follow-up period, we did not conduct further follow-up. Vaccination began in Japan in 2021. Therefore, there were no vaccine data before six months of follow-up. We investigated the vaccination status of the eight people who corresponded to the survey one year later (2021) for reference purposes. Of the six participants with a long-term anti-N IgG antibody response, two had been vac-
A subject who did not visit the institute two months later but did visit four months later. Positive: anti-nucleocapsid protein (anti-N) antibody-positive.

**b) Subclinical COVID-19 survey in 2021**

The participant flow is indicated in Fig. 2. Among the 332 survey participants, 5 (1.5%) tested positive for the anti-N IgG antibody. The subclinical COVID-19 survey in 2021 showed no items with a significant difference between the anti-N IgG antibody-positive and anti-N IgG antibody-negative groups (Table 2). The duration from the vaccination date to the testing date in the five anti-N IgG antibody-positive participants is shown in Table 3. Assuming that infection could be prevented after vaccination, a long-term anti-N IgG antibody response was observed for up to 95 days in participants who were subclinically infected with the novel coronavirus prior to their first vaccination and for up to 74 days in those infected prior to their second vaccination.

Furthermore, among the 10 physicians and nurses who tested positive for the anti-N IgG antibody in the 2020 survey, 9 participated in the 2021 survey. Only one of those participants had a positive result in the present survey. That participant tested negative for the antibody two months after the first testing during the 2020 survey.

**Discussion**

**Proportion of individuals with a long-term antibody response**

The present study showed that, according to a survey conducted 1 year after anti-N IgG antibody detection, 18.2% of participants who tested positive for the antibody still had the antibody (continuous presence) 1 year later. No long-term continuous anti-N IgG antibody response over one year has been reported in cases of subclinical COVID-19 infection, although a few long-term reports have been published concerning patients with symptomatic COVID-19. As in the present study, a study showed that only 36% of 367 subjects in Finland had a long-term anti-N IgG antibody response at 13 months after the infection based on the assessment of the anti-N IgG antibody (8). An 11-month survey in England reported that around half of individuals had undetectable anti-N IgG antibody levels eight months after initial seroconversion (9). A study in Thailand observed a long-term anti-N IgG antibody response in 26.6% of the 531 subjects (10). In addition, given the above findings, the proportion of individuals with a long-term antibody response to subclinical COVID-19 may be lower than among those with symptomatic COVID-19.
Table 1. Participant Characteristics of the Cases with the Long-term Response of Anti-N IgG Antibodies and Those without the Long-term Response of Antibodies until 12 Months after the Detection of Antibody Positivity.

|                                      | positive (n=6) | negative (n=27) | p      |
|--------------------------------------|---------------|-----------------|--------|
| Sex                                  |               |                 |        |
| Male                                 | 2 (33.3%)     | 11 (40.7%)      | 1.000  |
| Female                               | 4 (66.7%)     | 16 (59.3%)      | 0.098  |
| Age (y)                              | 73.7±9.6      | 60.9±17.6       | 0.215  |
| Body height (cm)                     | 156±7.3       | 160.6±8.2       | 0.384  |
| Body weight (kg)                     | 63.5±9.9      | 59.4±10.2       | 0.302  |
| Body mass index (kg/m²)              | 26±3.1        | 23±3.0          | 0.032† |
| Sleep duration (h)                   | 7.2±0.4       | 6.6±1.1         | 0.200  |
| Smoking habit                         |               |                 |        |
| Smoker                               | 0 (0%)        | 1 (3.7%)        | 1.000  |
| Previous smoker                      | 1 (16.7%)     | 6 (22.2%)       |        |
| Non-smoker                           | 5 (83.3%)     | 20 (74.1%)      |        |
| Drinking habit                        |               |                 |        |
| Drinker                              | 0 (0%)        | 14 (51.9%)      | 0.027* |
| Previous drinker                     | 0 (0%)        | 3 (11.1%)       |        |
| Non-drinker                          | 6 (100%)      | 10 (37%)        |        |
| BCG vaccination                      | 6 (100%)      | 24 (88.9%)      | 1.000  |
| Overseas travel in 2020              | 0 (0%)        | 0 (0%)          |        |
| Contact with overseas travelers or travelers who visited Japan in 2020 | 0 (0%) | 3 (11.5%) | 1.000 |
| Individuals infected with the novel coronavirus in the living environments such as home, workplace, school, and other places | 0 (0%) | 1 (3.7%) | 1.000 |
| History of influenza in 2020         | 0 (0%)        | 0 (0%)          |        |
| Use of air purifiers at home         | 2 (33.3%)     | 7 (26.9%)       | 1.000  |
| Use of trains 5 times a week or more | 0 (0%)        | 6 (22.2%)       | 0.563  |
| Presence of symptoms from January 2020 to the time of the first survey |         |                 |        |
| Cough                                | 1 (16.7%)     | 0 (0%)          | 0.182  |
| Runny nose                           | 0 (0%)        | 2 (7.4%)        | 1.000  |
| Sputum                               | 0 (0%)        | 1 (3.7%)        | 1.000  |
| Headache                             | 0 (0%)        | 1 (3.7%)        | 1.000  |
| Fever                                | 0 (0%)        | 0 (0%)          |        |
| Dysosmia                             | 0 (0%)        | 1 (3.7%)        | 1.000  |
| Dysgeusia                            | 0 (0%)        | 1 (3.7%)        | 1.000  |
| Vomiting                             | 0 (0%)        | 0 (0%)          |        |
| Diarrhea                             | 0 (0%)        | 1 (3.7%)        | 1.000  |
| Others                               | 1 (16.7%)     | 2 (7.4%)        | 0.464  |
| Underlying disease                   |               |                 |        |
| Hypertension                         | 6 (100%)      | 7 (25.9%)       | 0.002* |
| Use of ARB                           | 4 (66.7%)     | 3 (11.1%)       | 0.011* |
| Use of ACEI                          | 1 (16.7%)     | 0 (0%)          | 0.182  |
| Dyslipidemia                         | 2 (33.3%)     | 9 (33.3%)       | 1.000  |
| Diabetes                             | 4 (66.7%)     | 6 (22.2%)       | 0.053  |
| Type 1 diabetes                      | 0 (0%)        | 0 (0%)          | 1.000  |
| Type 2 diabetes                      | 4 (66.7%)     | 6 (22.2%)       | 0.053  |
| Hyperuricemia                        | 1 (16.7%)     | 0 (0%)          | 0.182  |
| Cerebrovascular disease              | 0 (0%)        | 1 (3.7%)        | 1.000  |
| Heart disease                        | 1 (16.7%)     | 3 (11.1%)       | 1.000  |
| Thromboembolism                      | 0 (0%)        | 1 (3.7%)        | 1.000  |
| Lung disease                         | 0 (0%)        | 2 (7.4%)        | 1.000  |
| Liver disease                        | 1 (16.7%)     | 1 (3.7%)        | 0.353  |
| Kidney disease                       | 0 (0%)        | 1 (3.7%)        | 1.000  |
| Immunological disease                | 0 (0%)        | 2 (7.4%)        | 1.000  |
| Group                                |               |                 |        |
| Patient                              | 6 (100%)      | 17 (63%)        | 0.145  |
| Doctor/nurse                         | 0 (0%)        | 10 (37%)        |        |
| Site                                 |               |                 |        |
| Clinic                               | 5 (83.3%)     | 21 (77.8%)      | 1.000  |
| Hospital                             | 1 (16.7%)     | 5 (18.5%)       |        |
| Clinic/hospital                      | 0 (0%)        | 1 (3.7%)        |        |

Data are mean±SD or n(%).

Data on the nominal scale were tested using the Fisher’s exact test, and continuous variables were tested using the Student’s t-test.

No subjects have been vaccinated (these participant characteristics are at the time of the first survey from May to June 2020).
Variation in the proportion of individuals with long-term antibody response depending on the target

The status of the long-term antibody response varies depending on the target antigen being recognized. In the report from Finland mentioned above, researchers found that NAb against the wild-type (WT) virus persisted in 89%, whereas anti-S IgG antibody was noted in 97% of subjects for at least 13 months after infection. These values were approximately 3 times the value for anti-N IgG antibody (36%) (8).

A study of 393 subjects during convalescence, including those with mild, moderate, and subclinical COVID-19, in France reported that the half-lives of the anti-S-RBD protein IgG antibody and anti-N IgG antibody were 725 [95% confidence interval (CI): 623-921] days and 283 (95% CI: 231-349) days, respectively (11). A study of 40 subjects in the United States also reported that the anti-N IgG antibody tended to persist for a shorter period than the anti-S protein IgG antibody (12). Since those results suggest that the anti-N IgG antibody may persist for a shorter period than the NAb, anti-S protein IgG antibody, and anti-S-RBD protein IgG antibody, care must be taken not to confuse these antibodies during evaluations.

A long-term survey for more than a year also reported that a longer antibody response was observed when conducted tests other than those for the anti-N IgG antibody. In a survey of 32 patients with mild and moderate symptomatic COVID-19 in Italy, a long-term anti-S-RBD protein IgG response was observed at 14 months in 96.8% (31 of 32 subjects) (13). In a study of 620 subjects in the United States, the adjusted predicted probability of positive NAb testing was 83% at 13 months (14). The presence of a long-term antibody response should be evaluated adequately after identification of the target antigen to be recognized.

Differences in the long-term antibody response depending on the severity of COVID-19

As mentioned at the beginning of this section, the proportion of individuals with a long-term anti-N IgG antibody response in the present study was lower than that in other studies of patients with symptomatic COVID-19. The proportion of patients with severe COVID-19 showing a long-term antibody response was previously reported to be higher than that of patients with a mild condition.

In a study of 532 Chinese subjects (82.1% with subclinical COVID-19), the NAb-positive proportions at 9 months after the diagnosis of COVID-19 were 63.6% among subjects with symptomatic COVID-19, 40.7% among those with subclinical COVID-19, and 46.0% among the overall population (15). Another survey in China showed that the proportion of NAb-positive individuals was 95.8% (183 out of 191 subjects) at 9 months after infection in 215 patients with a mild/severe condition (16). The duration of a certain level of strength of a humoral immune response varies depending on the severity of the inciting infection (17).

A survey in the United States indicated that adjusted predicted probability values of positive NAb tests at 13 months were 71% for clinics, 75% for emergency rooms, and 84% for hospitals, suggesting that the proportions of individuals with a long-term antibody response increased with severity (14). A survey of 236 Belgian participants indicated that the proportions with a positive anti-N IgG antibody response at 6-8 months were 38.9% among patients with a mild condition and 70.6% among those with a severe condition (18).

Based on these previous findings, the present results suggest that the proportion of subjects positive for an anti-N IgG antibody response at 1 year among individuals with subclinical COVID-19 was 18.2%. This lower value than
Table 2. Participant Characteristics of the Cases with the Response of Anti-N IgG Antibodies and Those without in 2021.

|                      | Positive (n=5) | Negative (n=327) | p    |
|----------------------|---------------|-----------------|------|
| Sex                  |               |                 |      |
| Male                 | 2 (40%)       | 100 (30.6%)     | 0.645|
| Female               | 3 (60%)       | 227 (69.4%)     |      |
| Age (y)              | 60±16.3       | 49.8±12.6       | 0.074|
| Body height (cm)     | 163±4.5       | 161.9±8         | 0.762|
| Body weight (kg)     | 63.4±12.1     | 59±11.7         | 0.401|
| Body Mass Index (kg/m²) | 23.8±4.3   | 22.4±3.5        | 0.368|
| Sleep duration (h)   | 5.8±0.8       | 6.1±1           | 0.500|
| Smoking habit        |               |                 |      |
| Smoker               | 0 (0%)        | 25 (7.6%)       | 1    |
| Previous smoker      | 1 (20%)       | 60 (18.3%)      |      |
| Non-smoker           | 4 (80%)       | 242 (74%)       |      |
| Drinking habit       |               |                 |      |
| Drinker              | 1 (20%)       | 151 (46.3%)     | 0.548|
| Previous drinker     | 0 (0%)        | 21 (6.4%)       |      |
| Non-drinker          | 4 (80%)       | 154 (47.2%)     |      |
| BCG vaccination      | 5 (100%)      | 293 (89.9%)     | 1    |
| Use of air purifiers at home | 2 (40%) | 132 (40.5%) | 1 |
| Use of trains 5 times a week or more | 1 (25%) | 80 (24.5%) | 1 |
| Underlying disease   |               |                 |      |
| Hypertension         | 0 (0%)        | 49 (15%)        | 1    |
| Use of ARB           | 0 (0%)        | 31 (9.5%)       | 1    |
| Use of ACEI          | 0 (0%)        | 2 (0.6%)        | 1    |
| Dyslipidemia         | 0 (0%)        | 47 (14.4%)      | 1    |
| Diabetes             | 1 (20%)       | 16 (4.9%)       | 0.232|
| Type 1 diabetes      | 1 (20%)       | 3 (0.9%)        | 0.039|
| Type 2 diabetes      | 1 (20%)       | 11 (3.4%)       | 0.169|
| Hyperuricemia        | 0 (0%)        | 6 (1.8%)        | 1    |
| Cerebrovascular disease | 0 (0%) | 0 (0%) | NA |
| Heart disease        | 0 (0%)        | 8 (2.4%)        | 1    |
| Thromboembolism      | 0 (0%)        | 1 (0.3%)        | 1    |
| Lung disease         | 1 (20%)       | 9 (2.8%)        | 0.143|
| Liver disease        | 0 (0%)        | 4 (1.2%)        | 1    |
| Kidney disease       | 0 (0%)        | 0 (0%)          | NA   |
| Immunological disease | 1 (20%) | 4 (1.2%) | 0.074|
| Group                |               |                 |      |
| Patient              | 2 (40%)       | 120 (36.7%)     | 1    |
| Doctor/nurse         | 3 (60%)       | 207 (63.3%)     |      |
| Site                 |               |                 |      |
| Clinic               | 2 (40%)       | 213 (65.1%)     | 0.366|
| Hospital             | 3 (60%)       | 112 (34.3%)     |      |
| Clinic/hospital      | 0 (0%)        | 2 (0.6%)        |      |
| SARS-CoV-2 vaccination | 5 (100%)    | 319 (97.6%)     | 1    |

Data are mean±SD or n(%).

Data on the nominal scale were tested using the Fisher’s exact test, and continuous variables were tested using the Student’s t-test.

Except for the SARS-CoV-2 vaccination and the result of anti-N IgG antibodies response, the data are based on the data at the time of the first survey in 2020.

Table 3. The Numbers of Days from Vaccination Date to Testing Date for Participants Who Tested Positive for the Anti-N IgG Antibody.

|                   | No. of days from the first vaccination date to the testing date | No. of days from the second vaccination date to the testing date |
|-------------------|---------------------------------------------------------------|---------------------------------------------------------------|
| Participant 1     | 29                                                            | 9                                                             |
| Participant 2     | 26                                                            | 5                                                             |
| Participant 3     | 87                                                            | 66                                                            |
| Participant 4     | 95                                                            | 74                                                            |
| Participant 5     | 83                                                            | 56                                                            |

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Previously reported in patients with symptomatic COVID-19 may be an appropriate result.

**Other reports regarding the long-term antibody response**

Other reports have described the long-term antibody response at least six months after an infection. For example, a study in Germany reported that reactivity to the virus spike protein was observed in 87% of individuals 10 months after infection (19). In a survey of 546 participants in Italy, IgG seroconversion was observed two months after infection in 90% of participants but decreased to 47% at 10 months (20). This difference may be associated with differences in the evaluation method and geographical region.

Regarding survey results at approximately 7 months after infection, another survey in Germany that included patients with mild and moderate COVID-19 showed that the anti-S protein IgG antibody could still be detected at approximately 7-8 months in 90% of patients (21). A survey in China reported that serum IgG (recombinant antigen containing the nucleoprotein and a peptide from the spike protein of SARS-CoV-2) was positive in approximately 80% of patients who had had a severe condition at approximately 7 months after the onset of symptoms related to COVID-19 (22). A survey in Qatar reported that natural infection with the novel coronavirus strongly protected against reinfection with the virus, with approximately 95% efficacy for at least 7 months (23). The anti-N IgG antibody-positive proportion at 6 months in our previous report was about 24%, which was lower than the IgG antibody prevalence in the above 3 studies. The prevalence of a long-term antibody response might be longer in patients with symptoms than in those without symptoms.

**Characteristics of participants with a long-term antibody response**

In the present survey, a comparison between the groups with and without a long-term antibody response indicated that the BMI, morbidity of hypertension, and usage rate of ARBs were higher in participants without drinking habits in the long-term antibody response group. Since the BMI was not considered a significant factor by a multivariate analysis, the results of the present survey are similar to those of our previous survey up to six months after infection.

The present findings suggest that antibody production is difficult to sustain if a patient drinks alcohol routinely. Simou et al. reported that the risk of community-acquired pneumonia rises to 8% with an increase in daily alcohol consumption for every 10-20 g (24). Another study reported that alcohol might impair immunity (25-27). Kageyama et al. reported that frequent alcohol consumption was associated with a reduction in the serum SARS-CoV-2 anti-S antibody titer after vaccination (28). Since the antibody titer of the vaccine is evaluated based on the S protein, there may be a difference in assays based on the N protein. However, our study showed the same tendency. Alcohol may affect the continued production of antibodies to SARS-CoV-2.

In addition, more cases had hypertension and used ARBs in the group showing continued antibody production than in the group not showing continued antibody production. SARS-CoV-2 binds to ACE 2 receptors present on the surface of cells for intrusion (2). Some studies suggest that ACE inhibitors and ARBs may prevent aggravation of COVID-19 (29-31). However, another paper found no significant difference in this respect (32). If a patient uses ARBs internally and more ACE2 receptors are produced, it may aid in maintaining antibody prevalence. However, other studies have reported that, in most cases, antibody prevalence continued longer as severity increased. Further verification is thus required with large-scale and long-term studies.

**Association of antibody positivity with novel coronavirus infection**

Six patients with continued antibody response were not diagnosed with COVID-19 during the one-year follow-up period. Krutikov et al. reported that the presence of anti-N IgG antibodies was associated with a substantially reduced risk of reinfection for up to 10 months after primary infection (33). In the present study, we examined the proportions of anti-N IgG antibody positivity by qualitative testing, but no deterministic antibody titer by which no breakthrough infection occurs was identified. Breakthrough infections with the novel coronavirus were reported even in patients who maintained high neutralizing antibody values (34, 35). Given that it is difficult to compare the results of studies using different reagents and that the virus can undergo mutations, the basic level of neutralizing antibodies necessary cannot be determined.

**Actual status of subclinical COVID-19 in 2021**

We concurrently conducted a follow-up with a survey of subclinical infection with the novel coronavirus from May to July 2021 in physicians and nurses, showing that the proportion with anti-N IgG antibody positivity was 1.5%. The survey that was performed with a follow-up in 2020 indicated that these proportions were 2.4% among overall participants and 2.0% among only physicians and nurses (4). These results suggest that subclinical COVID-19 has not spread to medical institutions. To our knowledge, there is no survey regarding subclinical COVID-19 that was conducted only in 2021. These findings suggest that Japanese medical institutions may have been able to enact thorough preventive measures for COVID-19, including vaccination.

**Limitations**

Several limitations associated with the present study warrant mention. The exact time of infection is unknown because subjects were patients with asymptomatic infections. Accordingly, the actual period of continued antibody production is also unknown. In addition, we did not determine titer values. Therefore, a quantitative assessment could not
be conducted, and our study results cannot be simply compared with those of other studies. Although it is meaningful to observe changes in IgG antibody titers in chronological order, there was no general-purpose kit for evaluating antibody titers at the beginning of this study. However, the IgG qualitative kit was considered sufficient for evaluating the presence of subclinical infection. We investigated the alcohol habits but were unable to determine the amount of alcohol intake. In addition, since there were relatively few young subjects in this study (4), we cannot discuss them in detail. We also cannot discuss mutant strains.

Conclusions

The proportion of individuals (6 of 33) with detectable antibodies at 12 months was lower than that of patients who developed and recovered from symptomatic COVID-19. The antibody tended to persist in the participants who used ARBs and who did not have a drinking habit. Furthermore, the 2021 survey for subclinical COVID-19 in physicians and nurses indicated a positive proportion of anti-N IgG antibody in 1.5%, suggesting no spread of subclinical infections.

The authors state that they have no Conflict of Interest (COI).

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