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[ORIGINAL ARTICLE]

Study on Continuation of Antibody Prevalence Six Months after Detection of Subclinical Severe Acute Respiratory Syndrome Coronavirus 2 Infections

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1 **Abstract**

2 **Objective**

3 To examine the continuation of antibody prevalence and background factors in antibody-
4 positive subjects after asymptomatic infection with severe acute respiratory syndrome
5 coronavirus 2 (SARS-CoV-2).

6 **Methods**

7 A study was carried out to investigate the SARS-CoV-2 antibody (IgG) prevalence. SARS-
8 CoV-2 antibodies (IgG) were measured and analyzed with immunochromatographic tests.

9 **Patients**

10 Among 1603 subjects, comprising patients, physicians, and nurses at 65 medical institutes in
11 Kanagawa, Japan, 39 antibody-positive subjects received follow-up for 6 months.

12 **Results**

13 Of the 33 subjects who consented to the follow-up (23 patients and 10 medical professionals),
14 continued positivity of IgG antibodies was confirmed in 11 of 32 cases (34.4%) after 2 months,
15 8 of 33 (24.2%) after 4 months, and 8 of 33 (24.2%) after 6 months. A significant difference
16 was found in the sleeping time, drinking habits, hypertension, and use of angiotensin-2 receptor
17 blockers on comparing subject background characteristics among three groups: patients with
18 antibody production that continued for six months after the first detection of positivity, patients

1 in whom antibody production stopped at four months, and patients in whom antibody
2 production stopped at two months.

3 **Conclusions**

4 The continuation rate of IgG antibody prevalence was 24.2% at 6 months after the first
5 detection of antibody positivity in cases with asymptomatic coronavirus disease 2019 (COVID-
6 19) infections. This percentage is low compared with the antibody continuation rate in patients
7 who have recovered from symptomatic COVID-19 infection.

8

9 **Key words:** SARS-CoV-2 IgG antibody; immunochromatography; COVID-19; subclinical
10 infection; epidemiological survey

11

1 **Introduction**

2 Caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the coronavirus
3 disease 2019 (COVID-19) pandemic has affected 223 countries as of February 19, 2021. There
4 have been 109 million infections and 2.4 million deaths as of February 19, 2021 (1). Worldwide
5 vaccine developments aimed at stabilizing the situation have been building expectations, but
6 the duration of antibody prevalence that can be obtained from such vaccines remains a point to
7 be addressed.

8 There have been several reports thus far concerning the continuation of antibody
9 prevalence in patients who have contracted SARS-CoV-2, and opinions are divided. Some have
10 reported that antibody prevalence was maintained for four to six months (2-6), while others say
11 it diminished after a short period of time (7-9). However, the antibody titers reportedly increase
12 with the severity of the infection (2,4,7,10), with more severe cases continuing to produce
13 antibodies for a longer period of time than milder cases (4,5) while being unlikely to cause
14 aggravation after vaccination.

15 To what extent antibodies can be produced and how long they remain after the infection
16 in asymptomatic cases have been attracting attention. While differing results have been
17 reported concerning the continued production of antibodies generated by COVID-19, there are
18 no reports involving the long-term follow-up evaluation of the antibody prevalence following
19 subclinical infections. Of note, we previously reported that 2.4% of physicians, nurses,

1 outpatients, and medical examinees generated antibodies from subclinical infections (11).

2 We therefore investigated the presence of antibodies at two, four, and six months after
3 the detection of antibody positivity in patients who tested positive for antibodies in the first
4 test.

5

1 **Material and methods**

2 *Ethic approval and consent to participate*

3 This study was registered with the Clinical Trials Registry (<https://www.umin.ac.jp/>;
4 UMIN000040333; May 8, 2020) and performed in accordance with the study protocol, the
5 Declaration of Helsinki, and the Ethical Guidelines for Clinical Studies of the Japanese
6 Ministry of Health, Labor, and Welfare. This study was approved by the Ethics Review Board
7 of the Kanagawa Physicians Association (April 27, 2020). All participants provided their
8 written informed consent before participation.

9
10 *Study design*

11 This multi-center epidemiological study at 65 institutes in Kanagawa, Japan, enrolled patients
12 from May 18 to June 24, 2020. Subjects received follow-up until January 31, 2021.

13 The inclusion criteria were as previously reported (11). Subjects were those who tested
14 positive in the first anti-SARS-CoV-2 antibodies (IgG) test and consented to follow-up.

15
16 *Method*

17 Subjects were informed of the study in writing before giving their written consent and answered
18 questionnaires. Their blood was then collected for antibody testing. They were informed of the
19 need for follow-up if they tested positive in the first antibody test. After obtaining their written

1 consent, their blood was collected for antibody testing at two, four, and six months after the
2 detection of antibody positivity, with clinical symptoms recorded at each timepoint.

3 The study was terminated when subjects tested negative for antibodies or after six
4 months.

5

6 *Assay kit*

7 The Cica Immuno-test SARS-CoV-2 IgG was used (12). This is a reagent developed through
8 collaborative research by Professor Akihide Ryo of the Department of Microbiology,
9 Yokohama City University Graduate School of Medicine and Kanto Chemical Co., Inc., Tokyo
10 Japan, which detects human anti- SARS-CoV-2 antibodies (IgG) contained in the serum of
11 individuals infected with the novel coronavirus. The reagent is based on the principles of
12 immunochromatography.

13 The test was performed immediately after blood collection. In brief, when a serum
14 sample containing human anti-SARS-CoV-2 antibodies (IgG) is dropped on the sample pad of
15 the test device, human anti-SARS-CoV-2 antibodies (IgG) move on the test strip in the test
16 device and form complexes with goat anti-human IgG antibodies labelled with black particles
17 (black particle-labelled antibodies) contained in the conjugate pad. These complexes then move
18 to the membrane and bind to viral proteins fixed where a test line will appear. This accumulates
19 black particle-labelled antibodies, which form the test line. If human anti-SARS-CoV-2

1 antibodies (IgG) are not present in the serum, black particle-labelled antibodies do not
2 accumulate, and no test line appears. Whether human anti-SARS-CoV-2 antibodies (IgG) are
3 present or not, black particle-labelled antibodies also bind to rabbit anti-goat IgG antibodies
4 fixed where a control line appears. This accumulates black particle-labelled antibodies, and the
5 control line appears. The control line is an indicator of the normal development of the serum.

6 This assay kit was verified by the National Institute of Health Sciences on a
7 simultaneous performance evaluation test of an antibody assay kit against the new coronavirus,
8 and the maximum dilution factor for which all cases were positive was 64-fold (13). The
9 accuracy of this assay kit was equal to or better than that of many other assay kits.

10

11 *Survey items*

12 Information, such as age and sex obtained in the first survey (11), and the presence of clinical
13 symptoms and IgG antibodies at two, four, and six months after the detection of antibody
14 positivity, was collected.

15

16 *Endpoints*

17 The SARS-CoV-2 antibody (IgG) positivity rate was assessed two, four, and six months after
18 the first detection of antibody positivity. Subjects were divided into three groups for a
19 comparison: patients with antibody production that continued for six months after the first

1 detection of positivity, patients in whom antibody production stopped at four months, and
2 patients in whom antibody production stopped at two months.

3

4 *Statistical analyses*

5 The data were analyzed using the R software program, version 3.5.2 (R Foundation for
6 Statistical Computing, Vienna, Austria [<https://www.R-project.org/>]). When comparing the
7 patients' background characteristic, Fisher's exact test was used for nominal variables, and an
8 analysis of variance was used for continuous variables. If the answer to a questionnaire survey
9 item was unknown, the datum corresponding to it was excluded from statistical tests. The two-
10 sided significance level was 5%. Demographic characteristics are presented as the
11 mean \pm standard deviation or the number of cases (%).

12

1 **Results**

2 Figure 1 shows the participant flow. Of the 39 subjects showing antibody positivity in the
3 subclinical infection study, the 33 who consented to participate in the study received follow-
4 up. The continued production of antibodies was observed in 11/32 cases (34.4%) at 2 months,
5 8/33 (24.2%) at 4 months, and 8/33 (24.2%) at 6 months. Of the subjects showing continued
6 antibody production, two were found to have clinical symptoms, such as a cough, during the
7 follow-up.

8 Table 1 shows the participant characteristics of the three groups: patients with antibody
9 production that continued for six months after the first detection of positivity, patients in whom
10 antibody production stopped at four months, and patients in whom antibody production stopped
11 at two months. There were significant differences among the three groups in sleeping time,
12 drinking habit, hypertension, and use of angiotensin-2 receptor blockers (ARBs).

13 Seven of the 39 antibody-positive individuals in the initial test underwent polymerase
14 chain reaction (PCR) testing. However, all of them were PCR-negative. Even in cases where
15 the antibody test was positive, the possibility of current infection/onset could not be ruled out.
16 Therefore, in some cases, PCR tests were performed, whereas in others, the tests were refused
17 by public health centers/medical associations. Some patients did not receive a PCR test because
18 they had no symptoms and did not want to get the check. Patients who were asymptomatic at
19 the subsequent follow-up were required to pay for the antigen test and PCR test themselves.

1 For this reason, it was up to the subject to decide whether or not to carry out the test. However,
2 we continued to follow patients in whom antibody production had stopped, with no subsequent
3 reports of infection.
4

1 **Discussion**

2 Antibody positivity was maintained in 11/32 cases (34.4%) at 2 months after the detection, in
3 8/33 (24.2%) at 4 months after detection, and in 8/33 (24.2%) at 6 months after detection in
4 this study. We continued to follow patients in whom antibody production had stopped and
5 considered them to be free from infection, as they had no suggestive symptoms.

6 There have been several reports on the short-term continued antibody production after
7 subclinical infection. In a 2-month follow-up study in India, only 57 of 201 (28.36%) subjects
8 in whom antibody positivity had been previously detected showed IgG antibody positivity
9 when they were tested again 2 months later (9). Long et al. conducted an 8-week follow-up of
10 subclinical patients, noting that the rate of continued positivity was 60% (18/30) (8). In our
11 study, we found the rate of continued positivity was 34.4% at 2 months after the first detection
12 of antibody positivity. This result was in the middle of previously reported values. In the present
13 study, we followed cases for up to six months after the infection, an unprecedented follow-up
14 duration for subclinical infection case; we consequently noted a low rate of continued antibody
15 production.

16 Follow-up studies have produced diverse results in symptomatic patients who were
17 followed from the recovery phase onward. In a study in which quantitative tests were
18 performed with antibody titers, the titer values remained stable in most cases for three months
19 and were still sufficient for protecting from viruses even after five months, despite some

1 decrease (5). In a large-scale study in Iceland, the antiviral antibody titer values with two types
2 of pan-Ig antibodies increased for two months after the diagnosis of infections with quantitative
3 PCR, with such values maintained for up to four months after the diagnosis (2). In a study in
4 Chicago, IgG antibodies to SARS-CoV-2 receptor-binding domain (RBD) remained detectable
5 for 4 months in 75% of 24 subjects who had shown IgG antibody positivity at the outset (3).

6 Although not conducted to verify antibody titers, a UK study found that 89 of 11,052
7 medical professionals who had not had antibodies at baseline developed symptoms of COVID-
8 19 during a 30-week study, while none of the 1246 who had had antibodies at baseline
9 developed COVID-19 symptoms (6). Their report further suggests that antibody production
10 could continue for at least six months.

11 It is impossible to simply compare these reports with our study because ours dealt with
12 subclinical infections, and follow-up was started after the detection of antibody positivity.
13 However, of note, antibodies became undetectable after a short period of time, a point that was
14 presumably influenced by the severity of symptoms. The acquisition of high antibody titers
15 that drop slowly facilitates continued antibody production.

16 Regarding the acquisition of the high antibody titers, Chen et al. performed a study
17 involving 92 subjects with symptomatic COVID-19 in the US, noting a significant correlation
18 between the volume of anti-SARS-CoV-2 IgG and the age and self-claimed severity (4). Other
19 studies reported similar results (2,7). According to Long et al., IgG production continued for a

1 longer time in symptomatic patients than in subclinical patients (8). Researchers in Japan also
2 reported that the virus-neutralizing antibody titers were found to be higher in more severe cases
3 than in milder ones, with more cytokines and chemokines produced in more serious patients
4 (10). These findings are supported by other study reports (14,15). Goto et al. also reported that
5 a regression analysis indicated that a high body mass index, a fever, and the need for
6 mechanical ventilation or extracorporeal membrane oxygenation were significantly associated
7 with elevated neutralizing antibody titers (16). There is likely a correlation between the degree
8 of virus-neutralizing antibody titers and the seriousness of symptomatic cases when antibodies
9 are produced. The antibody titers in our study were probably low because the cases were
10 asymptomatic.

11 Antibody production reportedly continued for a longer duration in cases that recovered
12 quickly despite the seriousness at the time of infection than other cases (4). Furthermore, in a
13 study from New York, when antibody titers were assessed in patients who had recovered from
14 COVID-19, the titers dropped more slowly in patients with higher titers during the recovery
15 phase, and the group with low titers included some who even showed antibody negativity by
16 three months after the onset of symptoms (5). In a study on subclinical infections in India, 8 of
17 243 patients complained of symptoms suggesting COVID-19 infections within 1 month after
18 the first confirmation of antibody positivity. These patients took PCR tests and were all
19 diagnosed with COVID-19. At that time, antibody production was not detected in seven of

1 these eight cases (9). Other studies have also suggested that antibodies tend to decrease if the
2 initial antibody production is low (17-21).

3 Considering the factors contributing to higher titer values and continued high titers, it
4 seems reasonable to say that the antibody production continuation ratio is low in cases with
5 subclinical infections compared with symptomatic cases.

6 While there have been a range of results reported, it is difficult to simply compare these
7 varied results because the testing methods are not standardized. In a previous study (8) in which
8 virus antibody responses stopped 8 weeks later in 40% of subjects, the antibodies were
9 measured using different methods from those in another study in which antibody production
10 continued for a long time (5). This is due in part to the former study targeting NPs and a single
11 linear epitope in spike proteins. Stable virus-neutralizing antibody titers were reported in a
12 study in which the antibody production was found to stop early. Therefore, the mechanism is
13 considered to depend on the target antigens. The results from a study in Chicago suggested a
14 greater sensitivity of RBD IgG assays than other assays, as the test results differed between
15 nucleocapsid and RBD IgG in the presentation of serum antibody positivity (3). Chia et al.
16 investigated the performance of N, S1, and RBD proteins from SARS-CoV-2 and SARS-CoV
17 using four different test platforms and reported that although RBD protein showed the greatest
18 specificity, the N proteins of both viruses had very high cross-reactivity and were unsuitable
19 for the detection of virus-specific antibodies (22). An N-terminal region that is highly

1 homologous with other viruses was deleted from the N protein used as the antigen in the
2 immunochromatographic kit used in the present study. Therefore, our kit will naturally produce
3 fewer false-positive results than other kits (23). It is necessary to closely check the testing
4 methods when interpreting study results.

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

14 In addition, more patients had hypertension and used ARB in the group showing
15 continued antibody production than in the group not showing the continuation of antibody
16 production. SARS-CoV-2 binds to angiotensin-~~converting enzyme (ACE)~~-2 receptors present
17 on the surface of cells for intrusion (29). Some studies have suggested that ~~ACE~~angiotensin-
18 converting enzyme (ACE) inhibitors and ARBs may prevent aggravation of COVID-19 (30-
19 32), but another paper reported that they didn't affect (33). If a patient uses ARB internally and

1 more ACE2angiotensin-2 receptors are produced, it may aid in maintaining antibody
2 prevalence. However, other studies have reported that, in most cases, antibody prevalence
3 continued for a longer period as the severity increased. Further verification is required with
4 large-scale and long-term studies.

5 Results concerning the relationship between antibody titers and sex vary by study,
6 although we found no marked differences by sex in the duration of antibody production. Some
7 studies have suggested that men tend to produce IgG antibodies with high antibody titers
8 (2,7,34), while another study reported opposite results (35). Further results are expected in the
9 future.

10 If vaccines are developed, the challenge would be the continuation of the antibody
11 production effect. The lack of continuous immunity to seasonal coronaviruses has also been
12 shown epidemiologically (36,37). It would be important to sustain the immune response of
13 both T and B cells (38).

14

15 **Limitation**

16 Several limitations associated with the present study warrant mention. The exact time of
17 infection is unknown because the subjects were patients with asymptomatic infections.
18 Accordingly, the actual period of continued antibody production is also unknown. In addition,
19 we did not determine the titer values. Therefore, a quantitative assessment was not possible,

1 and our study results cannot be simply compared with other studies. Although it is meaningful
2 to observe changes in IgG antibody titers in chronological order, there was no general-purpose
3 kit for evaluating antibody titers available at the beginning of this study. However, the IgG
4 qualitative kit was considered sufficient for evaluating the presence or absence of subclinical
5 infection. Finally, while we investigated the alcohol habits, we were unable to investigate the
6 amount of alcohol intake.

7

8 **Conclusions**

9 The IgG antibody production rate was 24.2% (8 of 33 cases) among patients with asymptomatic
10 infections of SARS-CoV-2 at 6 months after the first detection of antibody positivity. This was
11 lower than the rate among patients who recovered from symptomatic infections. In addition,
12 there were significant differences in drinking habit, hypertension, and the use of ARBs among
13 three groups: patients with antibody production that continued for six months after the first
14 detection of positivity, patients in whom antibody production stopped at four months, and
15 patients in whom antibody production stopped at two months. The duration of the continuation
16 of antibody production is a point that remains to be explored in vaccine development.

17

18 **Availability of data and material**

19 The biochemical data used to support the findings of this study are available from the

1 corresponding author upon request.

2

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16 **Contributions**

17 Conceived and designed the experiments: IM, NH, HD, MN, AK. Enrolled patients: IM, TT,
18 NH, MT, YW, NT, SK, YM, TN, TK, YO, AK. Analyzed the data: IM. Wrote the first draft of
19 the manuscript: IM, AK. Contributed to the writing of the manuscript: IM, AK. All authors

1 agreed with the manuscript's results and conclusions. All authors have read and confirm that
2 they meet the ICMJE criteria for authorship.

3

4 **The authors state that they have no conflicts of interest (COIs).**

5

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8 Prefecture Medical Association, and Kanagawa Prefecture.

9

10 **Figure legend**

11 **Figure 1. Participant flow.** *A patient who did not visit the institute two months later but did
12 visit four months later.

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