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MAJOR ARTICLE

The Antibody Response to SARS-CoV-2 Infection

Linda Hueston,[1] Jen Kok,[1] Ayla Guibone,[1] Damien McDonald,[1] George Hone,[1] James Goodwin,[1] Ian Carter,[1] Kerri Basile,[1] Indy Sandaradura,[1,2,3] Susan Maddocks,[1,2] Vitali Sintchenko,[1,3] Nicole Gilroy,[2] Sharon Chen,[1,3] Dominic E. Dwyer,[1,3] and Matthew V. N. O'Sullivan[1,2,3]

[1]New South Wales Health Pathology, Institute of Clinical Pathology and Medical Research, Westmead, Australia, [2]Department of Infectious Diseases, Westmead Hospital, Westmead, Australia, and [3]Sydney Medical School, The University of Sydney, Sydney, Australia

Background. Testing for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)–specific antibodies has become an important tool, complementing nucleic acid tests (NATs) for diagnosis and for determining the prevalence of coronavirus disease 2019 (COVID-19) in population serosurveys. The magnitude and persistence of antibody responses are critical for assessing the duration of immunity.

Methods. A SARS-CoV-2-specific immunofluorescent antibody (IFA) assay for immunoglobulin G (IgG), immunoglobulin A (IgA), and immunoglobulin M (IgM) was developed and prospectively evaluated by comparison to the reference standard of NAT on respiratory tract samples from individuals with suspected COVID-19. Neutralizing antibody responses were measured in a subset of samples using a standard microneutralization assay.

Results. A total of 2753 individuals were eligible for the study (126 NAT-positive; prevalence, 4.6%). The median "window period" from illness onset to appearance of antibodies (range) was 10.2 (5.8–14.4) days. The sensitivity and specificity of either SARS-CoV-2 IgG, IgA, or IgM when collected ≥14 days after symptom onset were 91.3% (95% CI, 84.9%–95.6%) and 98.9% (95% CI, 98.4%–99.3%), respectively. The negative predictive value was 99.6% (95% CI, 99.3%–99.8%). The positive predictive value of detecting any antibody class was 79.9% (95% CI, 73.3%–85.1%); this increased to 96.8% (95% CI, 90.7%–99.0%) for the combination of IgG and IgA.

Conclusions. Measurement of SARS-CoV-2-specific antibody by IFA is an accurate method to diagnose COVID-19. Serological testing should be incorporated into diagnostic algorithms for SARS-CoV-2 infection to identify additional cases where NAT was not performed and resolve cases where false-negative and false-positive NATs are suspected. The majority of individuals develop robust antibody responses following infection, but the duration of these responses and implications for immunity remain to be established.

Keywords. antibody; COVID-19; diagnosis; SARS-CoV-2; serology.

The acute respiratory tract disease coronavirus disease 2019 (COVID-19) caused by the novel coronavirus severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerged in Hubei province, China, in December 2019. As of May 21, 2020, there were more than 4.8 million cases worldwide. Diagnosis is primarily by detecting SARS-CoV-2-specific RNA by nucleic acid testing (NAT), but this has limitations, including the possibility of false-negative results due to low viral load in patients with minimal disease, inadequate respiratory tract sampling or mutations in the target sequence, and false-positive results due to contamination or nonspecific amplification.

Assays for detection of SARS-CoV-2-specific antibodies in serum or plasma can be used to confirm a diagnosis of COVID-19 or to make a retrospective diagnosis in individuals who have already recovered from acute illness and are no longer NAT positive [1], which can be critical for outbreak investigations [2]. Such assays also permit estimates of the proportion of a population who have been infected by testing unbiased collections of sera in population-weighted serosurveys. In addition, serology assays are needed to establish the effectiveness and durability of immune responses to SARS-CoV-2 infection for correlating humoral immune responses with disease severity [3], for facilitating studies of convalescent plasma and hyperimmune globulin as therapeutic or prophylactic interventions [4], and for investigating vaccine strategies.

The objective of this study was to develop and evaluate an immunofluorescent antibody (IFA) test for SARS-CoV-2-specific immunoglobulin G (IgG), immunoglobulin M (IgM), and immunoglobulin A (IgA) and apply it to document the serological response in individuals with confirmed COVID-19.

METHODS

Patient Selection and Sample Collection

Since the start of the epidemic in Australia, the Public Health Laboratory Network recommended collecting acute and convalescent sera for serological assays on individuals being tested

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Correspondence: M. O'Sullivan, MBBS, MMed, PhD, DTM&H, FRACP, FRCPA, NSW Health Pathology, ICPMR, Westmead, Locked Bag 9001, Westmead, 2145, Australia (matthew.osullivan@health.nsw.gov.au).

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for SARS-CoV-2 infection, in addition to respiratory tract samples for NAT, though this has not been universally adopted [5]. Individuals with suspected SARS-CoV-2 infection having both respiratory tract samples for NAT and serum samples for serological testing referred to the public health laboratory at the NSW Health Pathology-Institute for Clinical Pathology and Medical Research, Westmead, from January 22 to May 6, 2020, were prospectively included in this study. In addition, discarded blood samples collected for routine biochemistry from patients with NAT-confirmed COVID-19 managed at Westmead Hospital were utilized as individual seroconversion panels. A specificity panel consisting of samples positive for rheumatoid factor (n = 18), human influenza A virus (n = 18), or *Mycoplasma pneumoniae* (n = 8) antibodies collected during June–August 2019 were used to separately assess cross-reactivity.

SARS-CoV-2 Nucleic Acid Detection

Detection of SARS-CoV-2 RNA was performed on respiratory tract samples and viral culture supernatant using established methods [6, 7].

Viral Culture and Antigen Preparation

SARS-CoV-2 isolated from a sample collected on January 24, 2020, from an individual who acquired COVID-19 in Wuhan was utilized for the serological assays. The isolate belonged to SARS-CoV-2 linage A using the Phylogenetic Assignment of Named Global Outbreak Lineages Tool (Pangolin [8]); the consensus genome sequence has been submitted to GISAID (Accession EPI_ISL_407893 [9]). The virus was inoculated into Vero-E6 cells and examined daily for cytopathic effect (CPE) in a BSL-3 laboratory. Growth of SARS-CoV-2 was confirmed by the presence of CPE and the detection of SARS-CoV-2 RNA by NAT on culture supernatant. For IFA, infected cells were trypsinized at 36–40 hours postinfection and washed 3 times in phosphate buffered saline (PBS), before being fixed and permeabilized with acetone in wells on glass microscope slides.

SARS-CoV-2 IFA

Before detection of IgA and IgM, sera were pretreated with antihuman IgG (Eurosorb, Euroimmun, Leubeck, Germany) according to the manufacturer's instructions to remove IgG, which may compete with other antibody classes for binding sites. Sera were diluted 1:10 in PBS, added to the appropriate well on prepared slides, incubated at 37°C for 30 minutes, then washed before the addition of fluorescein-labeled antihuman IgG, IgM, or IgA (Dako, Denmark). After a further 30-minute incubation and washing, wells were examined using fluorescent microscopy, and a positive result was recorded if characteristic apple-green cytoplasmic staining patterns were identified (Figure 1). Samples positive at the initial screening dilution of 1:10 underwent repeat testing in serial dilutions to an end point antibody titer. Titers ≥10 were regarded as positive. Laboratory staff reading IFA results were unaware of NAT results but were aware of previous serology results for patients with paired samples.

SARS-CoV and MERS-CoV IFA

Samples positive for SARS-CoV-2 IFA underwent SARS-CoV and MERS-CoV IFA using commercially available slides (Euroimmun, Luebeck, Germany) according to the manufacturer's instructions.

SARS-CoV-2 Neutralizing Antibody Testing

Neutralizing antibody titers were determined by microneutralization using established methods [10].

Determination of the "Window Period"

Samples from individuals with NAT-confirmed SARS-CoV-2 infection that demonstrated seroconversion by IFA were used to determine the "window period" for appearance of SARS-CoV-2-specific antibodies. Only cases where the time period between the last negative and first positive samples was <48 hours were analyzed for this purpose. The time of seroconversion was recorded as the time of collection of the first positive sample, and the "window period" was calculated as the time from illness onset to time of seroconversion.

Positive Reference Standard

Positive reference cases were defined as persons with clinically suspected COVID-19 who had SARS-Cov-2 detected by NAT. Positive reference cases with an IFA titer of <10 beyond the observed upper range of the serologic window period were classified as having false-negative serology; otherwise they were

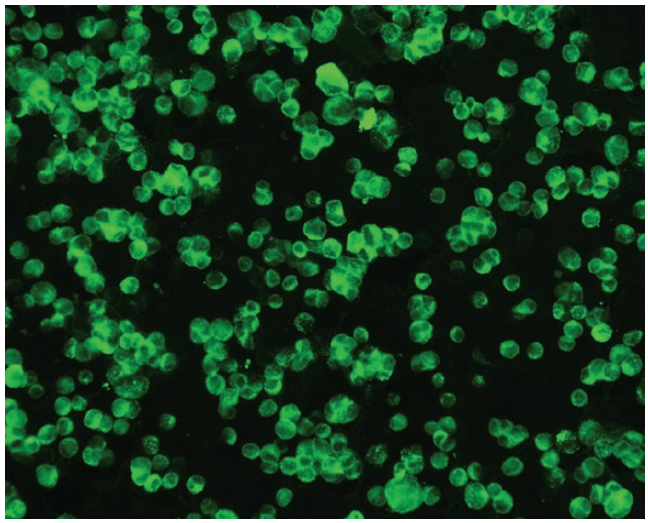

Figure 1. Positive immunofluorescent antibody test showing apple-green cytoplasmic fluorescence (1600× magnification).

classified as true positive if an IFA titer of ≥10 was detected at initial or follow-up testing.

Negative Reference Standard

Negative reference cases were defined as persons with suspected COVID-19 who had ≥1 negative SARS-CoV-2 NAT. Negative reference cases were classified as having false-positive serology if an IFA titer of ≥10 was detected on initial or follow-up serology; otherwise in these cases an IFA titer of <10 was classified as true negative.

Statistical Calculations

Sensitivity, specificity, negative and positive predictive values, and confidence intervals were calculated using medcalc (https://www.medcalc.org/). Confidence intervals for mean "window periods" were calculated using Microsoft Excel 2013.

RESULTS

Participants

Of 2753 individuals with suspected COVID-19 who were included in the study (Figure 2), 1685 (61.2%) were female, and the median age was 38 years with an interquartile range of 28–51. Two thousand five hundred seventy-seven individuals had a single serology test performed, and 176 had ≥2. One hundred twenty-six individuals were SARS-CoV-2 NAT positive (prevalence, 4.6%).

"Window Period"

Fifteen individuals had appropriately timed serum collections for calculation of the serological window period. The mean window period from symptom onset to seroconversion of ≥1 SARS-CoV-2-specific antibody class (IgG, IgA, or IgM) was 10.2 days (95% CI, 8.7–11.7) with an upper range of 14.4 days. Window periods for individual or combinations of antibody classes are shown in Table 1.

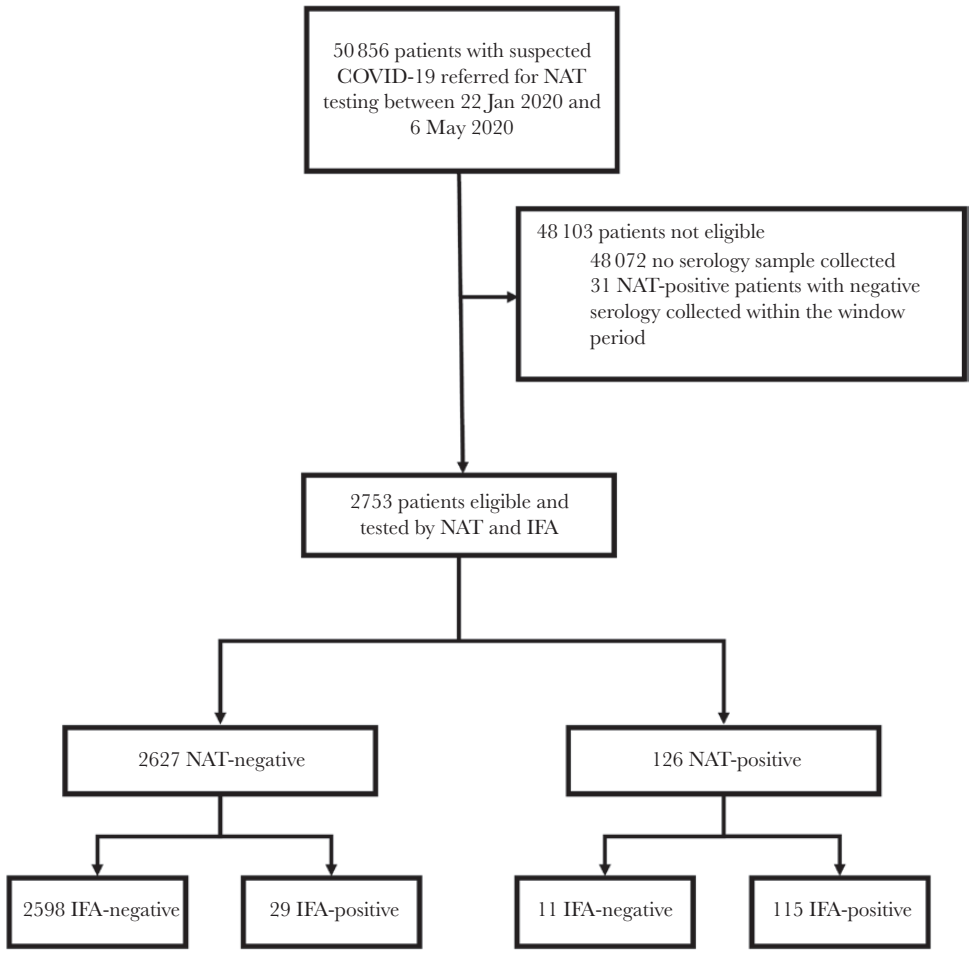

Figure 2. Study flow diagram. Abbreviations: IFA, immunofluorescent antibody; NAT, nucleic acid testing.

Table 1. Performance Characteristics of Antibody Detection by IFA for the Diagnosis of COVID-19

	Mean Window Period, d	Window Period Range, d	Sensitivity	Specificity	PPV	NPV
Any of IgG, IgA, IgM	10.2 (8.7–11.7)	5.8–14.4	91.3 (84.9–95.6)	98.9 (98.4–99.3)	79.9 (73.3–85.1)	99.6 (99.3–99.8)
All of IgG, IgA, and IgM	11.2 (9.6–12.7)	7.3–15.3	60.9 (51.1–70.1)	99.9 (99.7–100)	97.1 (89.3–99.3)	98.4 (98–98.7)
IgG	10.2 (8.7–11.7)	5.8–14.4	91.2 (84.8–95.5)	99.2 (98.7–99.5)	83.8 (77.3–88.7)	99.6 (99.3–99.8)
IgA	10.8 (9.3–12.3)	6.3–15.3	75.4 (66.8–82.8)	99.9 (99.6–100)	95.8 (89.6–98.4)	98.9 (98.5–99.2)
IgM	11.2 (9.7–12.7)	7.3–15.3	62.2 (52.5–71.2)	99.7 (99.4–99.8)	88.5 (79.7–93.7)	98.4 (98–98.8)
IgG and IgA	10.8 (9.3–12.3)	6.3–15.3	75.2 (66.5–82.6)	99.9 (99.7–100)	96.8 (90.7–99)	98.9 (98.5–99.2)
IgG and IgM	11.2 (9.7–12.7)	7.3–15.3	62.2 (52.5–71.2)	99.9 (99.7–100)	95.8 (88–98.6)	98.4 (98–98.8)
IgA and IgM	11.2 (9.6–12.7)	7.3–15.3	60.9 (51.1–70.1)	99.9 (99.7–100)	97.1 (89.3–99.3)	98.4 (98–98.7)

Values in parentheses are 95% confidence intervals.

Abbreviations: COVID-19, coronavirus disease 2019; IFA, immunofluorescent antibody; IgA, immunoglobulin A; IgG, immunoglobulin G; IgM, immunoglobulin M; NPV, negative predictive value; PPV, positive predictive value.

Sensitivity and Specificity

Of 126 NAT-confirmed cases, 115 had antibodies from 1 or more classes detectable by IFA within 14 days of illness onset (sensitivity, 91.3%; 95% CI, 84.9%–95.6%). The sensitivity, specificity, and negative and positive predictive values of individual antibody classes and their combinations are shown in Table 1.

Discordant Results

The 11 cases classified as false-negative serology results were predominantly from individuals with 1 positive and several negative NAT tests (Supplementary Table 1). When the 73 cases with only a single NAT-positive test were excluded, the calculated sensitivity of serology increased to 96.1% (95% CI, 86.5%–99.5%). Two individuals had multiple positive NATs but no SARS-CoV-2-specific antibody detected beyond the 14-day window period. One had severe COVID-19 and died on day 15 of illness, the second had mild disease with prolonged RNA shedding to day 26 of illness and no detectable antibody at day 17 of illness, but IgA detected at a titer of 10 on day 26.

Twenty-nine of 2627 NAT-negative patients had SARS-CoV-2-specific antibodies from 1 or more classes detectable by IFA (IgG [n = 22], IgA [n = 4], IgM [n = 9]) (Supplementary Table 2). Two of these were symptomatic household contacts of NAT-confirmed cases, and 2 further cases had a compatible clinical illness with single NAT-negative tests on days 12 and 20 after illness onset, respectively. In these 4 cases, false-negative NATs were strongly suspected. In the remaining 25 cases with false-positive IFA, the median antibody titers were lower (IgG, 40; IgA, 10; IgM, 10) compared with true-positive cases (IgG, 160; IgA, 30; IgM, 40) (Figure 3), and 24 had only 1 positive antibody class.

Neutralizing Antibody

Neutralizing antibody titers were measured in 97 samples from 32 individuals with NAT-confirmed SARS-CoV-2 infection. Seventy-two of the samples had antibody detected by IFA; all of these also had neutralizing antibody detected. There was a significant correlation between IgG and neutralizing antibody titers (r(95) = 0.27; P = .008) (Supplementary

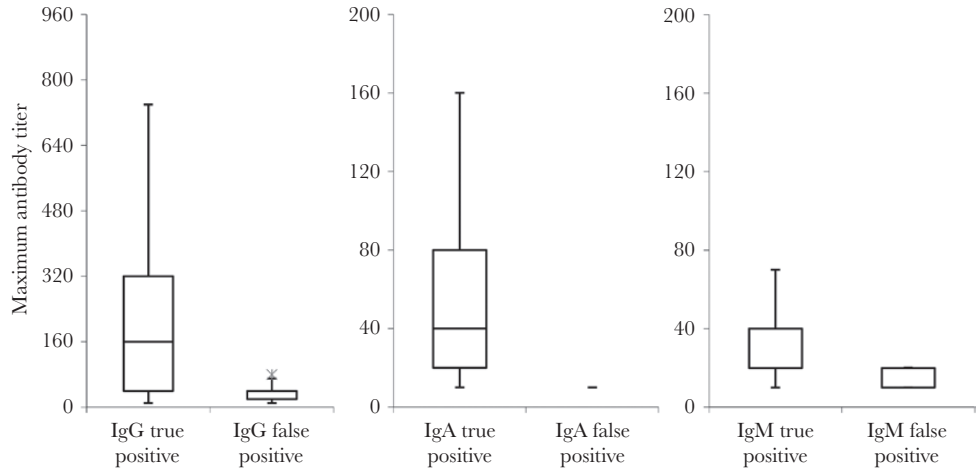

Figure 3. Boxplots of severe acute respiratory syndrome coronavirus 2–specific antibody titers seen in true-positive and false-positive immunofluorescent antibody tests. Abbreviations: IgA, immunoglobulin A; IgG, immunoglobulin G; IgM, immunoglobulin M.

Figure 1). Of the remaining 25 IFA-negative samples, 23 were collected within the first 14 days after illness onset and were negative for SARS-CoV-2-specific antibody by both IFA and neutralization. The 2 remaining samples were negative by IFA but had neutralizing antibody titers of 10 and 20 (collected at day 11 and day 12 after illness onset, respectively). Of 40 samples from 39 NAT-negative patients, 4 were positive by neutralization testing: These were from individuals in whom false-negative NAT tests were suspected (Supplementary Table 2).

Cross-reactivity

Nineteen samples with positive SARS-CoV-2 IgG, IgA, and IgM were tested for cross-reactivity using commercial SARS-CoV and MERS-CoV IFA tests. Cross-reactivity with SARS-CoV IgG was observed in 17 of 19 samples: All samples had a SARS-CoV-2 IgG titer of ≥160. Two samples showed cross-reactivity with SARS-CoV IgA, and 2 samples showed cross-reactivity with SARS-CoV IgM. In contrast, there was no cross-reactivity with MERS-CoV IgA or IgM. Two samples showed only borderline cross-reactivity with MERS-CoV IgG. None of the 37 samples from the specificity panel consisting of sera positive for rheumatoid factor, influenza A, or *M. pneumoniae* antibodies had detectable SARS-CoV-2 antibodies by IFA or neutralization.

Dynamics of the SARS-CoV-2-Specific Antibody Response

The median antibody titers in each of the 4-day intervals up to 28 days, followed by weekly intervals to 7 weeks, after illness onset were used to plot the dynamics of the antibody response using 425 samples from the 126 SARS-CoV-2-infected individuals (Figure 4). The peak antibody response was seen in the third week post–illness onset, with IgA and IgM titers declining

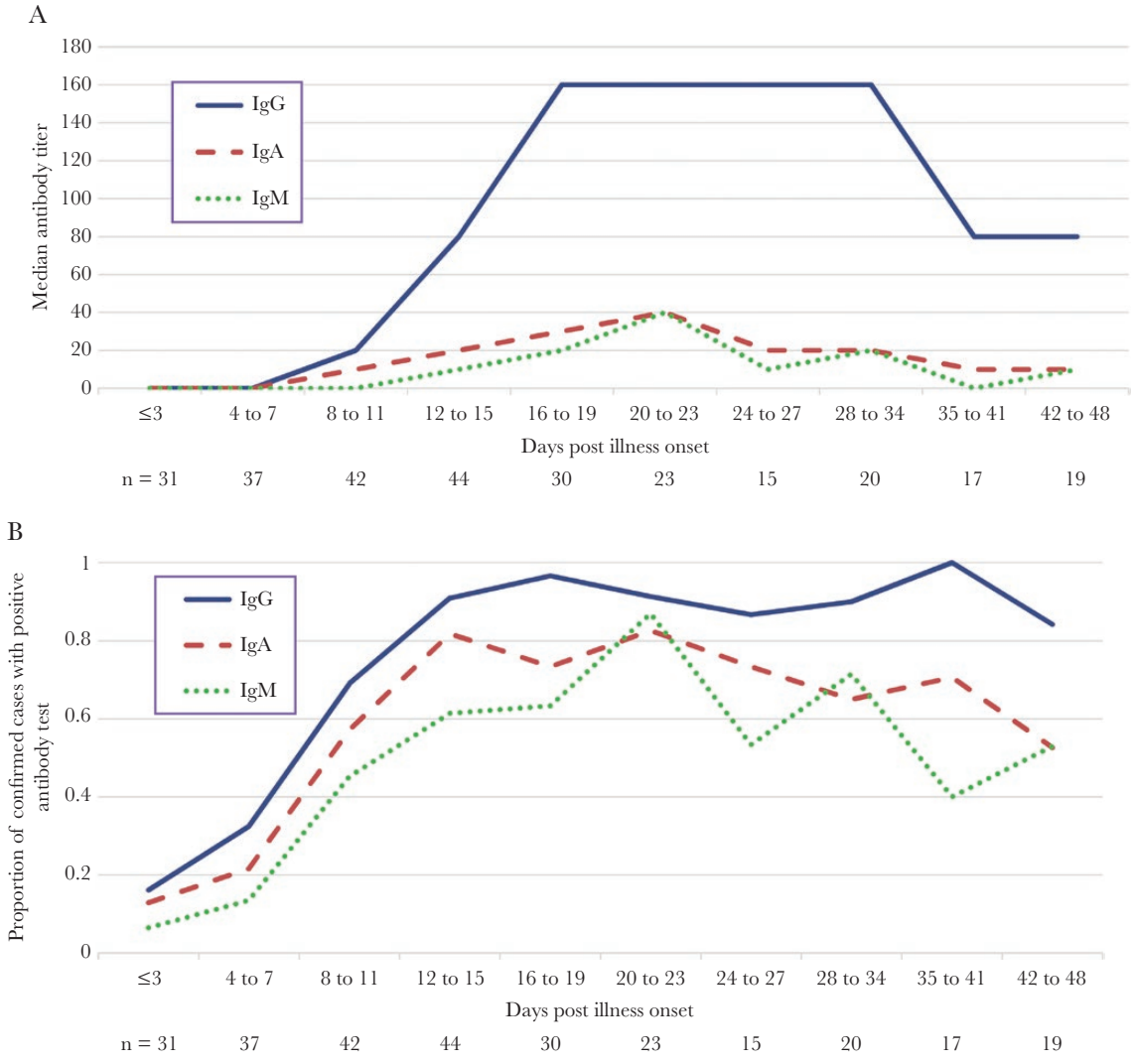

Figure 4. Dynamics of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) antibody response. A, Median antibody titers over time. B, Proportion of individuals with positive SARS-CoV-2-specific antibody results over time. Numbers below the axis indicate individuals tested at each time point. Abbreviations: IgA, immunoglobulin A; IgG, immunoglobulin G; IgM, immunoglobulin M.

after this time point, and IgG titers declining in the sixth week after illness onset. By the seventh week, the proportion of individuals who still had detectable IgG was 84%, but only 53% for IgA and for IgM. The maximum recorded IgG titer was, on average, higher in those who were hospitalized (486; 95% CI, 331–641) compared with those who were managed as an outpatient (179; 95% CI, 117–242; $P = .0001$). There was no correlation between antibody titers and sex, age, or duration of viral RNA shedding (Supplementary Figures 2–5).

DISCUSSION

We show that our in-house-developed IFA is a reliable diagnostic method for the detection of anti-SARS-CoV-2 antibodies. The sensitivity of this assay is greater, and window period shorter, than those reported for many other SARS-CoV-2 serology assays [1, 11–18]. This may be because the antigen utilized in this IFA assay is whole virion-infected cells rather than 1 or 2 purified or recombinant viral proteins. Complementing the high sensitivity is the high specificity of our IFA, with minimal cross-reactivity against coronaviruses other than SARS-CoV (also described in other studies [12, 16]). The absence of reactivity in the vast majority of cases without SARS-CoV-2 infection also suggests that cross-reactivity with common endemic coronaviruses such as CoV-229E and CoV-OC43 is minimal, as has been described for SARS-CoV [19]. False-positive cases were associated with low titers of 1 antibody class only. The IFA assay has the advantage of being quantitative, so changes in titer can be observed on paired samples, which aids in the interpretation of the results and helps time the onset of infection. Timing of sera collection after illness onset is important, and we recommend an acute sample in the first few days after illness onset and a second sample 14–28 days after illness onset to reliably detect seroconversion.

There may be a small but important incidence of false-positive SARS-CoV-2 NAT results, which are not always easily identified [20–24]. This can be particularly significant in jurisdictions where the incidence of COVID-19 cases is low. We recommend serological follow-up of unexpected NAT-positive cases in these circumstances in an attempt to confirm the diagnosis of COVID-19.

We found that SARS-CoV-2-specific IgA provided better performance characteristics than IgM, with higher sensitivity, equivalent specificity, and higher titers at all time points after illness onset. This is consistent with findings in other respiratory tract infections and has been observed previously for SARS-CoV-2 [25].

We chose to report any reactivity as positive in the IFA test, as the best way to resolve false-positive results is to collect a second sample to observe a rising titer. Low antibody titers that are associated with true-positive cases will generally demonstrate a rising titer on a second sample, while false-positive results generally show a low static or negative result on paired testing (data not shown).

This study suggests that the duration of SARS-CoV-2-specific IgA and IgM antibody responses is short lived, with frequent sero-reversion in the second month after illness onset. The majority of participants had detectable IgG by 48 days, albeit at lower titers. Longer follow-up is needed to determine the duration of IgG antibody responses in SARS-CoV-2 infection, but the waning IgG titers demonstrated in this study are in contrast to SARS-CoV and MERS-CoV infections, where the majority of cases have demonstrated strong antibody titers beyond 1 year postillness [26–28]. The duration of IgG persistence has implications for the timing of sample collection in individual cases, but also for population serosurveys where infections several months prior may not be captured if seroreversion has occurred. As with SARS-CoV, the appearance of IgG antibodies appears to be simultaneous with, or even occurs before, the appearance of IgA and IgM [13, 29].

While the association between serological response, humoral immunity, and protection from reinfection remains to be established for SARS-CoV-2, it is concerning that the kinetics of the antibody responses demonstrated in this study suggest that antibody responses may be short-lived, at least when measured by IFA. Whether this translates to early susceptibility to re-infection is a priority research area.

Investigations into the association of antibody response with disease severity in SARS-CoV-2, SARS-CoV, and MERS-CoV infections have yielded inconsistent results. Some studies have shown early robust antibody responses to be associated with mild disease [29, 30], while others have indicated that severe disease is associated with higher antibody titers, which may indicate a role for disease-enhancing antibodies in the pathogenesis of SARS-CoV-2 [11, 31–33]. Our study found that antibody titers were higher in individuals who were hospitalized for management of COVID-19. It is possible that certain antibody subsets are associated with protection against severe disease while others are associated with poorer outcomes [34]. Poorer immune responses may be associated with prolonged viral shedding, and the presence of antibody does not always correlate with viral clearance [35, 36]. We were unable to demonstrate associations between age, sex, or duration of viral shedding and antibody titer.

This study has several limitations. NAT was utilized as the reference standard for comparison to serology—false-positive and false-negative NATs may have resulted in underestimates of the sensitivity and specificity of IFA. Antibody testing by neutralization is a more appropriate reference standard but was only performed on a subset of samples due to the technical challenges associated with this method. Furthermore, samples classified as reference standard negative may have been collected from individuals who had an earlier undiagnosed SARS-CoV-2 infection but who were now presenting with a second unrelated

respiratory tract or febrile illness. Such individuals may have had negative NAT tests with persistent positive serology and would have been classified as false-positive serology tests. We did not assess for cross-reactivity with other endemic coronaviruses; however, if this were to be significant, we would have expected more false-positive results given that these other coronaviruses circulate commonly. The median age of participants was 38 years, with those at extremes of age being underrepresented, so our results may not be readily generalizable to these age groups.

IFA testing is a relatively specialized technique, and while robotic instruments can be utilized for slide preparation and incubation, the reading of results is a relatively manual process requiring well-trained laboratory staff. Commercially produced serology tests designed for high-throughput automated platforms such as chemiluminescent microparticle immunoassays and enzyme-linked immunosorbent assays may be more suitable than IFA for many laboratory settings but will need to be subject to robust evaluation.

CONCLUSIONS

Measurement of anti-SARS-CoV-2-specific antibody by IFA in serum is an accurate method for retrospective diagnosis of COVID-19. Serological testing should be selectively incorporated into diagnostic algorithms for SARS-CoV-2 infection for use in identifying additional cases where NAT was not performed and in helping resolve cases where false-negative and false-positive NATs are suspected. IFA and neutralizing antibody testing will serve as appropriate comparators for other SARS-CoV-2-specific antibody assays. This includes for assessment of rapid point-of-care antibody tests, which have been shown to be less sensitive and specific [37]. Future research is awaited to further define the duration of the antibody responses to SARS-CoV-2 infection and to understand the serological correlates of protection from re-infection. Antibody assays will play a major role in the understanding of COVID-19 epidemiology, pathogenesis, immunity, and immunotherapeutics.

Supplementary Data

Supplementary materials are available at Open Forum Infectious Diseases online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

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Potential conflicts of interest. All authors: no reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

Author contributions. L.H., D.D., and M.O'S. were responsible for study design. L.H., J.K., A.G., D.McD., G.H., J.G., I.C., K.B., V.S., and M.O'S. were responsible for data collection. All authors were involved in data interpretation. M.O'S. wrote the first draft of the manuscript, and all authors reviewed and approved the manuscript.

Patient consent. Individual patient consent was not required as this was an assay development and quality assurance study analyzing results from samples submitted for routine diagnostic testing. No patient samples were collected specifically for the purposes of this study, and results are presented in a de-identified manner. The study was approved by Health Protection New South Wales as a communicable diseases control activity.

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