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RESEARCH ARTICLE

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Discrepant serological findings in SARS-CoV-2 PCR-negative hospitalized patients with fever and acute respiratory symptoms during the pandemic

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Abstract

Coronavirus Disease 2019 (COVID-19) serology has an evolving role in the diagnosis of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection. However, its use in hospitalized patients with acute respiratory symptoms remains unclear. Hospitalized patients with acute respiratory illness admitted to an isolation ward were recruited. All patients had negative nasopharyngeal swab polymerase chain reaction (PCR) for SARS-CoV-2. Serological studies using four separate assays (cPass: surrogate neutralizing enzyme-linked immunosorbent assay [ELISA]; Elecsys: N-antigen based chemiluminescent assay; SFB: S protein flow-based; epitope peptide-based ELISA) were performed on stored plasma collected from patients during the initial hospital stay, and a convalescent visit 4–12 weeks later. Of the 51 patients studied (aged 54, interquartile range 21–84; 62.7% male), no patients tested positive on the Elecsys or cPass assays. Out of 51 patients, 5 had antibodies detected on B-cell Epitope Assay and 3/51 had antibodies detected on SFB assay. These 8 patients with positive serological test to COVID-19 were more likely to have a high-risk occupation ($p = 0.039$), bacterial infection ($p = 0.028$), and neutrophilia ($p = 0.013$) during their initial hospital admission. Discrepant COVID-19 serological findings were observed among those with recent hospital admissions and bacterial infections. The positive serological findings within our cohort raise important questions about the interpretation of sero-epidemiology during the current pandemic.

Abbreviations: A*STAR, Agency for Science, Technology and Research; ARI, acute respiratory illness; AUC, area under curve; COVID-19, coronavirus disease 2019; COI, cut-off index; CXR, chest X-ray; DSRB, Domain-Specific Review Board; ELISA, enzyme-linked immunosorbent assay; FDA, Food and Drug Administration; HCoV, human coronavirus; HRP-RBD, Horseradish peroxidase receptor binding domain; HSA, Health Sciences Authority; IRB, Institutional Review Board; IQR, interquartile range; LPS, lipopolysaccharide; MERS, Middle-East Respiratory Syndrome; MOH, Ministry of Health; OD, optical density; PCR, polymerase chain reaction; PI, propidium iodide; pio, postillness onset; PVA, polyvinyl alcohol; ROC, receiver operating characteristic; SARS, severe acute respiratory syndrome; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SD, standard deviation; SFB, S protein flow-based; SST, serum separating tubes.

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KEYWORDS
COVID-19, serology, Singapore

1 | INTRODUCTION

In early 2020, Singapore was not spared from the global Coronavirus Disease 2019 (COVID-19) pandemic.[1] There was the transmission of COVID-19 in the community and large outbreaks in migrant workers residing in dormitories.[2] Patients at risk of COVID-19 who were suspects by broad clinical criteria, were isolated. Once a polymerase chain reaction (PCR) positive case was identified, there was rapid contact tracing and quarantining of all exposed contacts.[3–5] In the early months of the pandemic, testing for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was limited to reverse (PCR)-based tests, while validated and widely accepted serological tests not being available until mid-2020.[6,7] Based on the experience from the SARS pandemic in 2003, hospitals were found to be particularly vulnerable. Indeed, while some centers managed nosocomial transmission of COVID-19 well,[8] nosocomial transmission of SARS-CoV-2 did contribute to the morbidity of this disease worldwide.[9–12]

Singapore's Ministry of Health (MOH) provided guidance on the criteria for any person to be considered a suspect case with COVID-19. Recognizing that the suspect case definition changed frequently as the epidemiological risk of the disease evolved over time, many public hospitals in Singapore extended isolation precautions to anyone who presented with a febrile illness and had symptoms consistent with an acute respiratory infection.[13,14] While such an abundance of caution was resource intense,[14,15] it helped identify cases early on and mitigated the risk of nosocomial transmission.[16]

Given the known high false-negative rate of PCR-based tests, particularly early in the disease,[17] we sought to determine what proportion of hospitalized patients with fever and/or respiratory symptoms were PCR negative yet serology positive for SARS-CoV-2 infection, using four separate serological assays, including two licensed assays and two experimental ones. Here, we describe discrepant serological findings among individuals hospitalized with fever and/or acute respiratory symptoms, during the early phase of the pandemic in Singapore and discuss the implications of these discrepancies on the diagnosis of COVID-19.

2 | METHODS

All participants in the hospitalized cohort were recruited with informed consent, and the study was approved by the Institutional Review Board of the National Healthcare Group, Singapore (2020/00194). Serum/plasma from COVID-19 patients recalled SARS patients, and healthy controls were obtained from other cohorts (DSRB 2012/00917, DSRB 2020/00091, CIRB 2017/2806, and NUS IRB 04-140).

2.1 | Study population

Patients aged 21 and above who were admitted to National University Hospital, Singapore between March 16, 2020, and June 19, 2020, were recruited. Patients had either fever, cough, coryza, sore throat, or shortness of breath and were isolated for evaluation of COVID-19. Due to national restrictions on the processing and analysis of biological samples from confirmed and suspected COVID-19 cases, patients who fulfilled the MOH's case definition of a suspect case for COVID-19 were excluded from the study. All patients had at least one nasopharyngeal PCR-negative swab at the entry to the study. Swabs were tested for SARS-CoV 2 by real-time PCR (RT-PCR) on the Roche cobas® platform at the hospital clinical laboratory. The detection of the ORF1ab gene target with or without the E-gene target was interpreted as a positive result. A single patient who did not undergo PCR testing was excluded from the analysis. Clinical information, including symptomatology, medical/drug history, results of investigations performed, demographic history, and history of risk factors for COVID-19 exposure, was collected at the first study visit (acute phase). Patients returned for a second study visit between 3 and 12 weeks later (convalescent phase).

Ten milliliters of blood was collected in BD Vacutainer serum separating tubes (SST) (Becton Dickinson) during both acute and convalescent phases. After clotting, serum was separated using centrifugation for 10 min at 1000 rcf, harvested, aliquoted into 500 µl, and stored at −80°C. Frozen aliquots were transferred with cold-chain maintained to testing laboratories in batches for the four assays listed below.

2.2 | Positive and negative controls

For the S protein flow-based (SFB) and Epitope assays, plasma was isolated from blood taken from 10 healthy volunteers who reported no intercurrent illness at the time of blood collection, no history of COVID-19 illness, and no known exposure to those with COVID-19. Ten individuals previously diagnosed with SARS-CoV during the outbreak in 2003 were contacted and serum samples were isolated as described above. Sera from 15 COVID-19 cases, who tested PCR-positive for SARS-CoV-2 via a nasopharyngeal swab, were used as positive controls. Patients were classified into three groups based on clinical severity: mild (no pneumonia on chest radiographs [chest X-ray, CXR] at baseline and during hospital admission; clinical severity 0), moderate (pneumonia on CXR without hypoxia; clinical severity 1), and severe (pneumonia on CXR with hypoxia (desaturation to 94%); clinical severity 2).

2.3 | Serological analysis

Assays were performed at laboratories within A*STAR Infectious Diseases Labs (Agency for Science, Technology and Research, A*STAR), Programme in Emerging Infectious Diseases (Duke-NUS Medical School) and Department of Laboratory Medicine, National University Hospital. Samples were analyzed in batches, with all assays run in duplicate. Calibration and quality control of the instruments used were performed to each manufacturer's recommendations.

2.3.1 | SARS-CoV-2 surrogate virus neutralization (cPass)

Before use, sera were heat-inactivated at 56°C for 30 min. SARS-CoV-2 Surrogate Virus Neutralization (cPass™) was performed using the test kit according to the manufacturer's recommendations (GenScript). Briefly, inactivated sera were pre-incubated with Horseradish peroxidase (HRP) conjugated recombinant SARS-CoV-2 receptor-binding domain (RBD) fragment (HRP-RBD). The mixture was added to a capture plate precoated with human ACE2 receptor protein (hACE2). Unbound HRP-RBD and HRP-RBD bound to non-neutralizing antibodies were captured on the plate, while circulating neutralization antibodies HRP-RBD complexes remained in the supernatant and were removed during washing. After washing, tetramethylbenzidine (TMB) solution was added followed by Stop Solution, with the final solution being read at 450 nm in a microtiter plate reader. The absorbance of the sample is inversely dependent on the titer of anti-SARS-CoV-2-neutralizing antibodies. A cut-off of >20% inhibition, as set by the manufacturer, was taken to be positive. This assay is approved for SARS-CoV-2 diagnosis by Singapore's Health Sciences Authority (HSA).

2.3.2 | Elecsys anti-SARS-CoV-2 serology test, Roche

The commercial Elecsys Anti SARS-CoV-2 assay uses a recombinant protein representing the nucleocapsid (N) antigen for the determination of antibodies against SARS-CoV-2. This test principle is that of a sandwich electro-chemiluminescent immunoassay.[18] Twenty microliters of sample are first incubated with biotinylated SARS-CoV-2-specific recombinant antigen and SARS-CoV-2-specific recombinant antigen labeled with a ruthenium complex, forming a sandwich complex. Upon addition of streptavidin-coated microparticles and a second incubation phase, the complex becomes bound to the solid phase via the interaction of biotin and streptavidin. The reaction mixture is then aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are subsequently removed with ProCell/ProCell M. Application of a voltage to the electrode then induces chemiluminescent emission, which is measured by a photomultiplier and is directly proportional to the analyte concentration. A signal sample/cut-off (COI) index is calculated, where a value of ≥1.0 indicates the presence of anti-SARS-CoV-2 antibodies. This assay is approved by both Singapore's Health Science's Authority and the US Food and Drug Administration (FDA).

2.3.3 | SFB assay

Before use, sera were inactivated with 10% Triton X-100 (Thermo Fisher Scientific) as above. The sera were screened for antibodies specific for the S protein as previously described.[19] S protein-expressing cells were seeded at 1.5×10^5 cells per well in 96-well V-bottom plates. The cells were first incubated with serum (diluted 1:100 in 10% fetal bovine serum [FBS]) before a secondary incubation with a double stain, consisting of Alexa Fluor 647-conjugated secondary antibodies (diluted 1:500) and propidium iodide (PI; diluted 1:2500). Secondary antibodies used are conjugated anti-human IgM or IgG. For assays examining IgG subclasses, the secondary incubation was with mouse anti-human IgG1, IgG2, IgG3, or anti-human IgG4. Following the secondary incubation, the cells were then incubated with Alexa Fluor 647-conjugated anti-mouse IgG. Cells were read on BD Biosciences LSR4 laser and analyzed using FlowJo (Tree Star). Data are shown as mean ± SD of two independent experiments, with dotted lines indicating mean + 3 SD of healthy donors. An isotype response was defined as positive by SFB assay when binding was more than mean + 3 SD of the healthy controls. The thresholds using the health control readings are based on the normal-like distribution of the healthy control reading where a mean + 3 SD would mean that there is a less than 0.13% chance of a false positive. Receiver operating characteristics (ROC) curves were previously constructed from each of the antibody binding with the healthy controls and SARS-CoV-2 patients as the true negatives and true positives respectively using the pROC library in R version 3.6.4.[19]

2.3.4 | B-cell epitope enzyme-linked immunosorbent assay (ELISA) assay

Before use, sera were inactivated with 10% Triton X-100 (Thermo Fisher Scientific), minimum 2 h at RT (final concentration: 1% Triton X-100). Inactivated sera were used at 1:100 dilution to screen against four linear epitopes on the SARS-CoV-2 Spike (S) protein: S14P5, S20P2, S21P2 and one epitope on the nucleocapsid (N) phosphoprotein: N4P5.[20,21] Briefly, Nunc Maxisorp flat-bottom 96-well plates (Thermo Fisher Scientific) were coated overnight with 50 µl per well of 0.5 µg/ml of NeutrAvidin protein (Thermo Fisher Scientific). Blocking was performed for 1 h with 0.01% polyvinyl alcohol (PVA; Sigma-Aldrich) in 0.1% PBST (blocking buffer). Peptide coating was performed at 1:2000 dilution for 1 h, followed by diluted inactivated sera for 1 h. Goat anti-human IgG (Jackson ImmunoResearch) secondary antibody was incubated for 1 h in blocking buffer at 1:1000 dilution. Development was performed with 50 µl of TMB

(Sigma-Aldrich), followed by 50 µl of 0.16 M sulfuric acid (Merck), with the final solution being read at 450 nm on an Infinite M200 plate reader (Tecan). Samples were tested by two independent experiments with the means of both taken to be true. Optical density (OD) values of samples were normalized to a positive control to account for plate-to-plate variations, and background signals were subtracted. A cut-off value above mean + 3 SD of healthy control samples was taken to be positive. The determination of the threshold of a positive result has been previously described.[21] Briefly, ROC curves for peptides to differentiate between SARS-CoV-2 infections and others were performed using the best thresholds determined as the maximum of the Youden's J statistic. Areas under the curve (AUC) were calculated for each peptide. For peptide combination analyses, logistic regression models were used to model the combinatory effects of 2, 3, and 4 peptides' OD readings toward the prediction of SARS-CoV-2 infection from others as a binary outcome. The logistic regression model fitted values were then used for ROC analysis to identify the optimal thresholds as well as AUCs. This was conducted using R version 3.6.2.

2.4 | Statistical analysis

Patient characteristics were examined, with continuous variables presented as the median and interquartile range (IQR), while categorical variables were presented as frequencies and percentages. Results of each serology test were tabulated against the initial and convalescent visit, by patient category (e.g., healthy controls, recovered SARS, acute COVID-19 illness, hospitalized cohort) and days postillness onset (pio). Characteristics of patients who tested positive for SARS-CoV-2 using any of the serological assays were described in greater detail, including their occupation and clinical presentation. Those with positive assay findings were then compared against those who tested negative using Student's *t* test for continuous variables, and χ^2 tests (or Fisher's Exact test where appropriate) for categorical variables. A p value of < 0.05 was considered significant. Data analyses were done using GraphPad Prism (GraphPad Software). All statistical analyses were performed using Stata 16.1 (StataCorp).

3 | RESULTS

3.1 | Overall population

Fifty-one patients were prospectively enrolled between March 16, 2020, and June 19, 2020, and their demographic details are presented in Table 1.

The median age of patients in our cohort was 54 years (range 21–84), 62.7% were male (39.2% Chinese, 29.4% Malay, 17.6% Indian). Healthcare workers (HCW) formed 12% of patients and 22.5% were nonhealthcare essential workers. 45.1% of patients had hypertension, 41.2% had dyslipidemia and 25.5% had diabetes mellitus. 86.3% and 70.6% of patients had fever or cough at

TABLE 1 Characteristics of study population

Demographic and clinical background	Overall (n = 51)
Median age (years)	54 (21–84)
Gender (male)	32 (62.7%)
Ethnicity	
Chinese	20 (39.2%)
Malay	15 (29.4.%)
Indian	9 (17.6%)
Others	7 (13.7%)
Occupation	
Healthcare worker	6 (12%)
Nonhealthcare worker	44 (88%)
High-risk profession*	12 (out of 45)
Unknown	1
Household size	
Median number of household size	3 (1–7)
Number with 5 or more in house	13
Medical history	
No past medical history	8 (15.7%)
Ischemic heart disease	9 (17.6%)
Hypertension	23 (45.1%)
Dyslipidaemia	21 (41.2%)
Diabetes mellitus	13 (25.5%)
Chronic kidney disease (including ESRF)	8 (15.7%)
Airways disease	9 (17.6%)
Clinical features	
Median number of days of first serology test from pio	5 (1–73)
Fever	44 (86.3%)
Median number of days with fever before presentation	2 (1–21)
Cough	36 (70.6%)
Median number of days with cough before presentation	3 (1–30)
Sore throat	14 (27.5%)
Malaise	5 (9.8%)
Dyspnea	17 (33.3%)
Coryzal symptoms	15 (29.4%)
Diarrhea	10 (19.6%)
CXR findings	(n = 50)
Normal CXR report	25 (51%)
Pneumonia	12 (23.5%)

(Continues)

TABLE 1 (Continued)

Demographic and clinical background	Overall ($n = 51$)
Abnormal, noninfective changes	13 (21.6%)
Median White Cell Count ($\times 10^9$/L)	9.61 (3.03–23.32)
Absolute neutrophil count ($\times 10^9$/L)	6.5 (0.45–20.93)
Absolute lymphocyte count ($\times 10^9$/L)	1.38 (0.17–5.45)
Patients with lymphocyte count <1.0	15 (28.8%)
C-reactive protein (CRP)	$n = 19$
Median CRP (mg/L)	97 (8–342)
Patients with CRP > 50	14 (73.7%)

Abbreviation: CXR, chest X-ray; ESRF, end stage renal failure.

presentation, respectively, with a median time of 2–3 days of symptoms before presentation. Only 29% of patients had lymphopenia ($<1.0 \times 10^9$/L). 24% of patients had pneumonia on CXR, and 51% of patients had a normal CXR report with the remainder (22%) having noninfective changes (Table 1).

Median days pio for acute sera to be taken was 5 (IQR 4) days and 34 (IQR15.5) days for convalescent sera. None of the enrolled patients had any contact with proven or suspect COVID-19 cases. They were not part of any confirmed clusters of COVID-19 in Singapore or had had contact with persons under quarantine. One patient had returned from overseas in March 2020, a few days before their hospital admission. While 80% of patients lived in Singapore at the time of the SARS epidemic, no patients reported having had SARS or Middle-East Respiratory Syndrome (MERS) in the past. Of the 51 patients in the study, 39 (76.5%) returned to have convalescent-phase sera collected. Between the first and the second study visits, none of the patients reported exposure to a confirmed COVID-19 patient or person in quarantine.

3.2 | Results from four serological assays

3.2.1 | cPass and Elecsys

The Elecsys and cPass assays showed that none of the patients, either during the acute or convalescent illness phases, had levels of SARS-CoV-2 specific antibodies that crossed the predefined threshold of positivity. Results from the cPass and Elecsys assays for the eight patients with positive serology results via the SFB assay and the B-Cell Epitope Assay are presented in Table 2.

3.3 | SFB assay

None of the patients was found to have total IgG or IgG3 against the S protein. However, three participants were found to have raised IgG1 at both the acute and convalescent visits against the full-length spike protein (Figure 1). The magnitude of binding for these three participants was above the predetermined positivity threshold but was lower than the median percentage binding seen in COVID-19 cases with mild disease previously.[19]

3.4 | B-cell Epitope assay

Five out of 51 (9.8%) patients had SARS-CoV-2-specific antibodies recognizing epitopes on the Spike (S) protein; S14P5, S20P2, and S21P2, and on the Nucleocapsid (N) protein; N4P5, as shown in Figure 1. One participant had antibodies that recognized two epitopes (S20P2, N4P5), while the remaining four participants had antibodies to single epitopes against SARS-CoV-2 (Table 3). Three participants had antibody titers, which increased from below the threshold of positivity to positive between the acute and convalescent visits. Two patients had positive antibody levels during the acute hospital visit, one of whom had antibody levels against N4P5-2, which approximated those of controls with moderate to severe COVID-19 disease. This patient's antibodies remained detectable at the convalescent visit on Day 34 pio, although it dropped below the set threshold for positivity (Table 2).

3.5 | Clinical characteristics of eight patients with positive peptide antibodies and SFB antibodies against SARS-CoV-2

The clinical and serological characteristics of these eight patients with various antibodies against SARS-CoV-2 is shown in Table 2. The median age of this group was 55 years with five males. Acute sera were collected between 1 and 10 days pio (median = 3), and convalescent sera were collected between 26 and 58 days pio (median = 35). None in the group were HCW, however, 5/8 (62.5%) patients were workers engaged in a high-risk occupation for COVID-19, which was significantly different from the proportion of those in the negative serology group (10/43, $p = 0.039$). Five patients with positive serology (62.5%) had a proven bacterial infection during their acute hospital admission, which was significantly different from the negative serology group (9/43, $p = 0.028$) (Table 3). A statistically significant difference was found between the proportions of patients who had positive serology with neutrophilia during the acute admission (5/8), compared with neutrophilia seen in the negative serology patients (7/43, $p = 0.013$) (Table 3).

4 | DISCUSSION

Since the advent of this study, serological assays have proven to be of great utility given the high proportions of COVID-19 patients who are asymptomatic, or who have subclinical infections.[22] In our study, we demonstrated that eight out of 51 (15.7%) patients admitted with a febrile illness or with acute respiratory illness (ARI), and with a negative swab PCR for SARS-CoV-2, were found to have low titers of specific antibodies against SARS-CoV-2, despite no known previous

TABLE 2 Clinical characteristics of patients with antibodies against SARS-CoV-2

P	V	pio	SFB (IgG1% inh)	Epitope (OD)		cPass (% inh)	Elecsys (COI)	Sex	Age	Occupation	Neut	Lymph	CXR	Diagnosis	Bacteria
1	1	10	1.07	S14P5 0.093		−1.05	0.083	F	27	Hotel receptionist	3.72	1.97	Normal	Pyrexia Unknown Origin	None
	2	58	0.82	**S14P5 0.194**		5.41	0.087								
2	1	5	**18.4**	N4P5 0.004		−4.22	0.082	M	46	Cab driver	19.44	1.38	Normal	Stump infection with osteomyelitis	*B. fragilis*
	2	26	**17.23**	N4P5 0.03		4.78	0.086								
3	1	3	**28.65**	S21P2 0.04		2.87	0.075	M	48	Container equipment specialist	11.88	0.73	Normal	Erythema Multiforme	None
	2	44	**26.23**	S21P2 0.06		10.40	0.086								
4	1	2	**19.67**	S21P2 0.03		0.87	0.083	M	78	Businessman	9.23	0.42	Normal	Bacteremia	*E. coli*
	2	35	**23.23**	S21P2 0.04		13.62	0.086								
5	1	5	1.37	**N4P5 0.845**		2.97	0.078	F	55	Teacher	13.9	1.18	Fluid overload/ Pneumonia	Infected intrauterine device	*B. fragilis*
	2	34	1.09	N4P5 0.436		10.66	0.082								
6	1	3	0.47	S14P5 0.147		7.23	0.081	M	54	Security guard	2.31	1.5	Normal	Pneumonia	None
	2	38	0.44	**S14P5 0.183**		−0.92	0.083								
7	1	1	2.00	S20P2 0.149	N4P5 0.266	−14.45	0.080	M	75	Retired	12.98	0.43	Pneumonia	Catheter-related bloodstream infection	*S. aureus*
	2	26	1.46	**S20P2 0.222**	**N4P5 0.533**	−1.46	0.086								
8	1	2	0.15	**S21P2 0.276**		4.19	0.074	F	58	Housewife	14.65	1.64	Normal	Prevertebral abscess	None

Note: Threshold for optical density (OD) = mean + 3 SD. Threshold for S14P5 = 0.170, S20P2 = 0.212, S21P2 = 0.237, N4P5 = 0.473. Results from positive serological assays are presented in bold red. For the Epitope assay, results shown are the epitopes with the highest antibody response at Visit 1 and Visit 2, or if they crossed the threshold to be considered positive.

Abbreviations: *B. fragilis*, *Bacteroides fragilis*; cPASS, SARS-CoV-2 surrogate virus neutralization assay expressed as percent inhibition; CXR, chest X-ray; *E. Coli*, *Escherichia coli*; Elecsys, anti-SARS-CoV-2 serology test by Roche expressed as a sample cut off index; ELISA, enzyme-linked immunosorbent assay; Epitope, B-cell Epitope ELISA assay expressed as OD; Lymph, lymphocyte count expressed in cells × 10^9/L; Neut, neutrophil count expressed in cells × 10^9/L; P, patient; pio, post illness onset; *S. aureus*, *Staphylococcus aureus*; SFB, S protein flow-based assay expressed as percent inhibition by IgG1; V, visit.

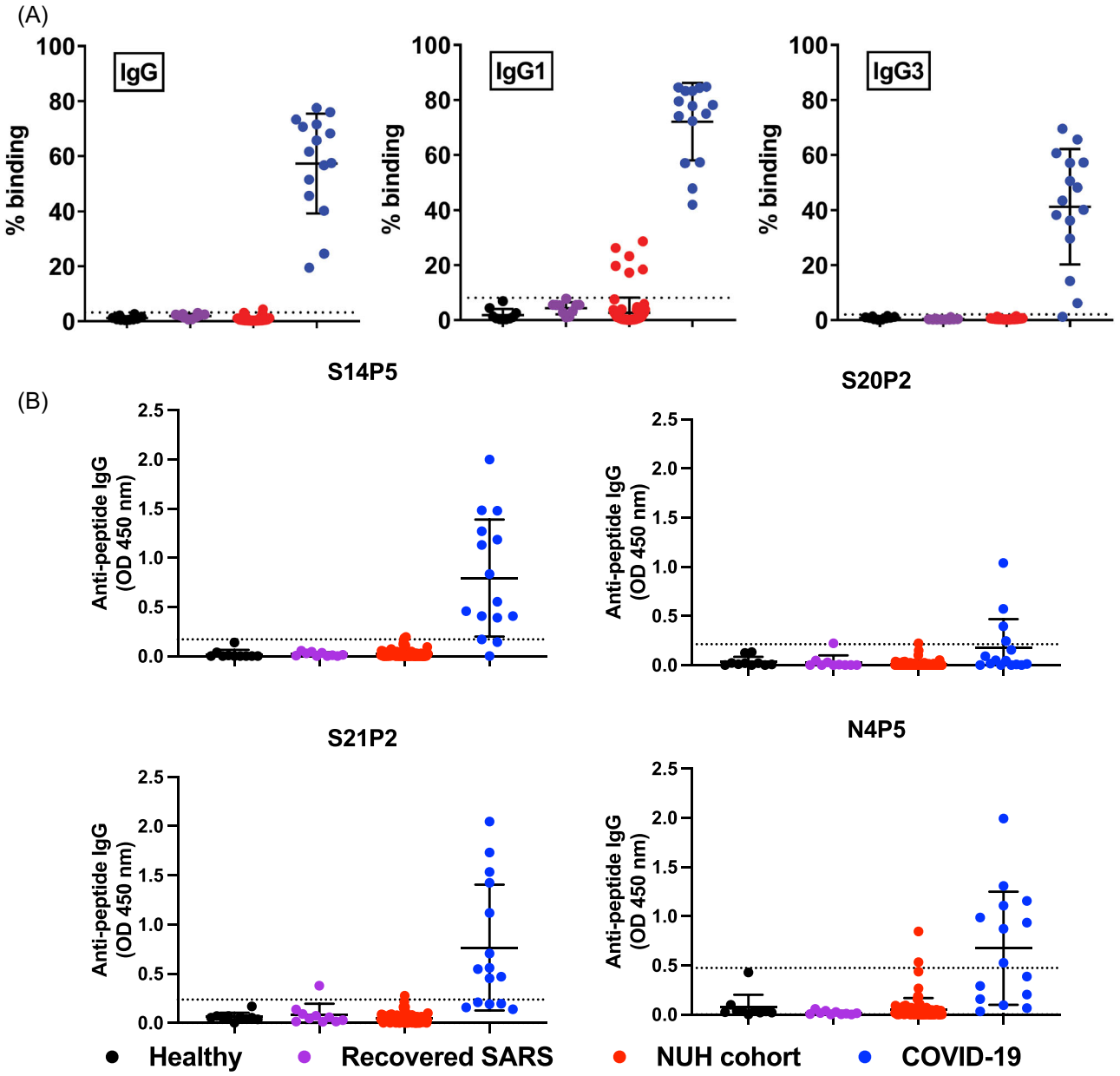

FIGURE 1 Serological analysis by S protein flow-based (SFB) and B-cell epitope assays. Sera from symptomatic patients (n = 51), isolated for evaluation of COVID-19, were collected at acute and convalescent (between 3 and 12 weeks later) timepoints. Serum samples were screened at 1:100 dilution (A) in an SFB assay for specific total IgG, IgG1, and IgG3 against full-length SARS-CoV-2 S protein expressed on the surface of HEK293T cells, and (B) in a peptide-based enzyme-linked immunosorbent assay (ELISA) against four IgG linear B-cell epitopes of SARS-CoV-2: spike S14P5, S20P2 and S21P2, and nucleocapsid N4P5. Sera or plasma samples from healthy donors (n = 22 for SFB; n = 10 for epitope assay), recovered SARS patients (n = 20 for SFB; n = 10 for epitope assay), and COVID-19 patients (n = 15; median 23 days postillness onset) were included as controls. Data are shown as mean ± SD of two independent experiments, with dotted lines indicating mean + 3 SD of healthy donors. An isotype response was defined as positive by SFB assay when the binding is more than mean + 3 SD of the healthy controls

exposure to COVID-19. Blood was collected between March and June 2020, before the development and release of any COVID-19 vaccine. Of the eight patients with positive antibodies against SARS-CoV-2, five patients had detectable antibodies during their hospital admission, with three patients who developed antibodies only at the convalescent visit (Table 2). All patients demonstrated the presence of antibodies against the same epitope or the same subclass at both visits, even if it was below the positive threshold (Figure 1). We explored possible explanations for the low-positive titers of SARS-CoV-2 antibodies found in these eight PCR negative patients.

4.1 | True exposure to SARS-CoV-2 virus

First, both the B-cell Epitope assay and the SFB assays have been shown to have a high sensitivity and specificity to SARS-CoV-2 at

TABLE 3 Patients with positive SARS-CoV-2 serology versus those with negative SARS-CoV-2 serology

Parameter	Serology positive, *n* = 8 (%)	Nonserology positive, *n* = 43 (%)	
Male	5 (62.5)	27 (62.8)	$p = 1.0$
Median age	54.5 (range 27–78)	52 (range 21–84)	$p = 0.44$
High-risk occupation	5 (62.5)	10 (23.3)	$p = 0.039$
Median number in household	3.5 (range 1–5)	3 (range 1–7)	
Presence of fever + ARI	6 (75)	31 (72.1)	$p = 1.0$
Pneumonia on CXR	2 (25)	12 (27.9)	$p = 1.0$
Patients with neutrophilia (>10.0)	5 (62.5)	7 (16.3)	$p = 0.013$
Patients with lymphopenia (<1.0)	3 (37.5)	12 (27.9)	$p = 0.67$
Any infection	7 (87.5)	31 (72.1)	$p = 0.66$
Bacterial infection	5 (62.5)	9 (20.9)	$p = 0.028$

Abbreviations: ARI, acute respiratory illness (e.g., cough, coryzal symptoms, sore throat, sputum production); CXR, chest X-ray; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

23 days pio (Table S1), which bolsters confidence that these results represent a true infection in 6/8 patients with positive serology at the convalescent visits. At least one patient produced antibodies against two epitopes S20P2 and N4P5-2 (Table 2), the combination of which was previously shown to have a sensitivity and specificity of 100%.[21] On the SFB assay, three patients had IgG1 binding to SARS-CoV-2 at both the acute and convalescent timepoints, with a specificity of 96.72% for SARS-CoV-2 (Table 3).

We found a statistically significant difference in the proportion of patients who had high-risk occupations for the acquisition of SARS-CoV-2 ($p = 0.036$, Table 3). These patients were a hotel receptionist, taxi driver, teacher, security guard, and another who worked as a container equipment specialist and thus visited construction sites where he had contact with migrant dormitory workers, the latter being a group who experienced a staggeringly high prevalence of SARS-CoV-2 infection at 56.1%[23,24] (Table 3). While none in the positive serology group were HCW, who are often perceived as the occupation group at highest risk of exposure, the other occupations represented within the positive serology group approximated that risk.[25] Titers of antibodies in the positive serology group were quantitatively lower than those from mild cases of COVID-19 our group has shown previously.[19,21] Since IgG levels in asymptomatic COVID-19 infection have been found to be significantly lower than those who develop symptoms,[26] the positive serology patients may have had asymptomatic COVID-19 infection, with the reason for hospitalization accounted for by other aetiologies.

However, the absence of a rise in the titer of antibodies from the acute to convalescent visit (Table 2) wherein the convalescent visit occurred at a median of 35 days from illness onset in these eight patients, is not supportive of true SARS-CoV-2 exposure. Instead, the persistent production of such antibodies suggests the presence of cross-reactive antibodies.

4.2 | Cross-reactivity to other coronaviruses

Five out of eight patients in the positive serology group were found to have antibodies against SARS-CoV-2 during the acute hospital visit, at a median of 3 days pio (range 2–5) (Table 3). Since the production of antibodies against SARS-CoV-2 typically takes days to weeks,[27] we considered the possibility that the results seen were due to cross-reactivity to other coronavirus infections.

SARS was responsible for more than 200 cases in Singapore in 2003.[28] Prior studies have shown cross-reactive binding between antibodies from SARS patients to SARS-CoV-2,[29,30] and both the B-cell Epitope and SBF assays were tested for cross-reactivity against sera collected from patients with previous SARS infection.[31,32] While no cross-reactivity was found, the literature on whether recovered SARS patients can sustain the production of SARS-CoV-specific antibodies is inconclusive.[30,32,33] It may be remotely possible that patients in the positive serology group had occult SARS infection, or failed to report SARS exposure or infection when asked. Similar cross-reactivity may be observed in other human coronaviruses (HCoV).[34,35] Coronaviruses have been found to result in heterologous boosting, where B-cell recall of a prior coronavirus infection could result in varied antibody production that may have specificity to more than one specific type of coronavirus. Such heterologous boosting of antibodies has been thought to account for the cross-reactivity of SARS-CoV-2 antibodies to endemic HCoV.[35]

4.3 | Polyclonal B cell activation from a bacterial infection

Seven out of the eight patients with SARS-CoV-2-specific antibodies were hospitalized with an infection, and the remaining patient presented with erythema multiform with an unknown trigger (Table 3). Based on chart review by an infectious diseases specialist, five patients had a confirmed bacterial infection: two patients cultured *Bacteroides fragilis* from an amputation stump wound and an infected intrauterine device, respectively, two patients had bacteremia with *Escherichia Coli* and *Staphylococcus aureus*, respectively, and one patient had a prevertebral abscess, which is typically caused by Gram-positive bacteria. Reasons for admission for the remaining three patients were community-acquired pneumonia, pyrexia of unknown origin, and erythema multiforme, all of which may have also been caused by a bacterial infection.

The presence of the bacterial infection has been previously shown to result in polyclonal B-cell activation and the formation of a multitude of antibodies with nonspecific binding properties.[36–38] Since a large proportion of patients with positive serology had proven or presumed bacterial infection, they may have had polyclonal B-cell activation resulting in the production of antibodies that had some cross-reactivity to the SARS-CoV-2 virus.

4.4 | Biological false-positive laboratory result

Despite the high sensitivity and specificities of both the B-cell Epitope assay and the SFB assay demonstrated previously[32] all patients in our cohort tested negative for SARS-CoV-2 antibodies on the two assays approved by the regulatory authorities (Elecsys, cPass), and none of the eight patients tested positive for both the Epitope peptide ELISA and SFB assay. The risk of a false-positive serology test increases as the prevalence of COVID-19 decreases in the community.[39] Based on positive PCR results, the prevalence of COVID-19 in Singapore at the time has been reported to be between 0.04% and 0.25%, depending on whether we rely on positive PCR results or the results of an unpublished sampling study conducted by the Singapore MOH.[40,41] Seroprevalence among household contacts of those with COVID-19 were found to be substantially higher at 5.5%[2] and seroprevalence among migrant workers living in dormitories was a log scale higher at 56.1%.[25] In our patient cohort, none of the patients reported being a contact of a COVID-19 case or had lived in a migrant workers dormitory setting although some had contact with migrant workers. Thus, the positive predictive value (PPV) for the B-cell Epitope and SFB assays is low for the low-risk community cases enrolled in our study, but the PPV would have been higher if used among high-risk groups such as in a migrant worker dormitory (Table S1).

5 | LIMITATIONS

This was a single-center small-sized cohort of patients of hospitalized patients with ARI. It was a heterogeneous cohort, and we only examined patients who were hospitalized and could not examine patients in isolation facilities outside of our hospital. The adoption of widespread vaccination would likely change the serological profile of the general population that is admitted to the hospital and alter the use of serology as a diagnostic tool in the context of SARS-CoV-2 infection.

6 | CONCLUSIONS

The spectrum of immunological responses to SARS-COV-2 infection is only slowly being elucidated. While we have confirmed that currently approved serological tests should be used for diagnostic purposes in the appropriate clinical settings, novel serological assays raise important questions about unusual aspects of the host response to this pathogen. In particular, we need to understand the possibility of cross-reactivity to other as yet unknown viruses, possibly immune activation from other infections which could explain consistently positive results on epitope assays, and be mindful of the use of serological testing in settings where the prevalence of COVID-19 is low.

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CONFLICTS OF INTEREST

A patent application for the SFB assay has been filed (Singapore patent #10202009679 P: A Method Of Detecting Antibodies And Related Products). A patent application for SARS-CoV-2 linear B-cell epitopes has been filed under the applications 10202002981 P and 10202004276 P. The remaining authors declare no conflicts of interest.

ETHICS STATEMENT

This study was approved by the hospital's institutional review board (National Healthcare Group (NHG) Domain Specific Review Board (DSRB) 2020/00194). Retrospective analyses were performed and non-identifiable, anonymized data are presented. Informed consent was obtained from each study participant before conducting the study. All study methods were carried out in accordance with relevant guidelines and regulations by the Declaration of Helsinki and DSRB. Individual participant information is not published in this manuscript.

AUTHOR CONTRIBUTIONS

Gail B. Cross and Jinghao N. Ngiam wrote the original draft of the manuscript. Gail B. Cross, Jinghao N. Ngiam, Claire M. Naftalin, Yun S. Goh, Siti N. Amrun, Paul A. Tambyah, Lin F. Wang, Laurent Renia, Dale Fisher, and Lisa F. P. Ng contributed to the revision of further drafts. Gail B. Cross, Claire M. Naftalin, Natasha Bagdasarian, Chek M. Poh, Yun S. Goh, Wan Ni Chia, Siti N. Amrun, Sai M. Tham, Hazel

Teng, Rawan Alagha, Shoban K. Kumar, and Shaun S. Y. Tan were involved in data collection and data analysis. Gail B. Cross, Claire M. Naftalin, Natasha Bagdasarian, Paul A. Tambyah, Lin F. Wang, Laurent Renia, Dale Fisher, and Lisa F. P. Ng were involved in the conception, data analysis, and critical review of the manuscript.

DATA AVAILABILITY STATEMENT

Data may be made available on reasonable request from the corresponding author.

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SUPPORTING INFORMATION

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