# Temporal profiles of viral load in posterior oropharyngeal saliva samples and serum antibody responses during infection by SARS-CoV-2: an observational cohort study

*Kelvin Kai-Wang To*, Owen Tak-Yin Tsang*, Wai-Shing Leung, Anthony Raymond Tam, Tak-Chiu Wu, David Christopher Lung, Cyril Chik-Yan Yip, Jian-Piao Cai, Jacky Man-Chun Chan, Thomas Shiu-Hong Chik, Daphne Pui-Ling Lau, Chris Yau-Chung Choi, Lin-Lei Chen, Wan-Mui Chan, Kwok-Hung Chan, Jonathan Daniel Ip, Anthony Chin-Ki Ng, Rosana Wing-Shan Poon, Cui-Ting Luo, Vincent Chi-Chung Cheng, Jasper Fuk-Woo Chan, Ivan Fan-Ngai Hung, Zhiwei Chen, Honglin Chen, Kwok-Yung Yuen*

## Summary

**Background** Coronavirus disease 2019 (COVID-19) causes severe community and nosocomial outbreaks. Comprehensive data for serial respiratory viral load and serum antibody responses from patients infected with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) are not yet available. Nasopharyngeal and throat swabs are usually obtained for serial viral load monitoring of respiratory infections but gathering these specimens can cause discomfort for patients and put health-care workers at risk. We aimed to ascertain the serial respiratory viral load of SARS-CoV-2 in posterior oropharyngeal (deep throat) saliva samples from patients with COVID-19, and serum antibody responses.

**Methods** We did a cohort study at two hospitals in Hong Kong. We included patients with laboratory-confirmed COVID-19. We obtained samples of blood, urine, posterior oropharyngeal saliva, and rectal swabs. Serial viral load was ascertained by reverse transcriptase quantitative PCR (RT-qPCR). Antibody levels against the SARS-CoV-2 internal nucleoprotein (NP) and surface spike protein receptor binding domain (RBD) were measured using EIA. Whole-genome sequencing was done to identify possible mutations arising during infection.

**Findings** Between Jan 22, 2020, and Feb 12, 2020, 30 patients were screened for inclusion, of whom 23 were included (median age 62 years [range 37–75]). The median viral load in posterior oropharyngeal saliva or other respiratory specimens at presentation was $5\cdot2$ $\log_{10}$ copies per mL (IQR $4\cdot1$–$7\cdot0$). Salivary viral load was highest during the first week after symptom onset and subsequently declined with time (slope $-0\cdot15$, 95% CI $-0\cdot19$ to $-0\cdot11$; $R^2=0\cdot71$). In one patient, viral RNA was detected 25 days after symptom onset. Older age was correlated with higher viral load (Spearman's $\rho=0\cdot48$, 95% CI $0\cdot074$–$0\cdot75$; $p=0\cdot020$). For 16 patients with serum samples available 14 days or longer after symptom onset, rates of seropositivity were 94% for anti-NP IgG (n=15), 88% for anti-NP IgM (n=14), 100% for anti-RBD IgG (n=16), and 94% for anti-RBD IgM (n=15). Anti-SARS-CoV-2-NP or anti-SARS-CoV-2-RBD IgG levels correlated with virus neutralisation titre ($R^2>0\cdot9$). No genome mutations were detected on serial samples.

**Interpretation** Posterior oropharyngeal saliva samples are a non-invasive specimen more acceptable to patients and health-care workers. Unlike severe acute respiratory syndrome, patients with COVID-19 had the highest viral load near presentation, which could account for the fast-spreading nature of this epidemic. This finding emphasises the importance of stringent infection control and early use of potent antiviral agents, alone or in combination, for high-risk individuals. Serological assay can complement RT-qPCR for diagnosis.

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*Contributed equally

**State Key Laboratory for Emerging Infectious Diseases, Carol Yu Centre for Infection, Department of Microbiology, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Pokfulam, Hong Kong Special Administrative Region, China** (K K-W To MD, C C-Y Yip PhD, J-P Cai BSc, L-L Chen MPhil, W-M Chan PhD, K-H Chan PhD, J D Ip MSc, A C-K Ng BSc, R W-S Poon PhD, C-T Luo MD, V C-C Cheng MD, J F-W Chan MD, Z Chen PhD, H Chen PhD); **Department of Clinical Microbiology and Infection Control, The University of Hong Kong–Shenzhen Hospital, Shenzhen, China** (K K-W To, J F-W Chan, K-Y Yuen MD); **Department of Medicine and Geriatrics, Princess Margaret Hospital, Hong Kong Special Administrative Region, China** (O T-Y Tsang FRCP, W-S Leung FRCP, J M-C Chan MPH, C Y-C Choi MRCP, D P-L Lau MRCP, T S-H Chik MRCP); **Department of Medicine, Queen Mary Hospital, Hong Kong Special Administrative Region, China** (A R Tam MRCP); **Department of Medicine, Queen Elizabeth Hospital, Hong Kong Special Administrative Region, China** (T-C Wu FRCP); **Department of Pathology, Queen Elizabeth Hospital, Hong Kong Special Administrative Region, China** (D C Lung FRCPath); **and Department of Medicine, Li Ka Shing Faculty of Medicine, The University of Hong Kong,**

## Introduction

Coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), was first reported from China in December, 2019.[1] Although Middle East respiratory syndrome coronavirus (MERS-CoV)[2] and severe acute respiratory syndrome coronavirus (SARS-CoV)[3] infections have a higher mortality rate than does COVID-19, SARS-CoV-2 spreads much more rapidly than MERS-CoV and SARS-CoV. Reliable data for profiles of serial viral load and serum antibody responses are needed urgently to guide antiviral treatment, infection control, epidemiological measures, and vaccination. The peak viral load of patients with MERS-CoV and SARS-CoV infections occurs at around 7–10 days after symptom onset, which could be associated with nosocomial outbreaks involving health-care workers.[2,4] Clinical studies of antiviral agents for SARS showed that the viral load decreased significantly with treatment success.[5] No systematic study of these two important variables with statistical analysis has been

Pokfulam, Hong Kong Special Administrative Region, China (I F-N Hung MD)

Correspondence to: Dr Kwok-Yung Yuen, Department of Clinical Microbiology and Infection Control, The University of Hong Kong–Shenzhen Hospital, Shenzhen, China
kyyuen@hku.hk

## Research in context

### Evidence before this study

We searched PubMed on Feb 24, 2020, with no limitations by starting date, with the terms "COVID-19", "coronavirus", "antibody", and "viral load"; we restricted our search to articles published in English. Our search did not retrieve any reports on clinical progression of coronavirus disease 2019 (COVID-19) with respect to temporal viral load and concomitant serum antibody profiles. We identified one correspondence piece on viral load with no statistical analysis, and another article with a few cases of antibody response.

### Added value of this study

We present findings of an observational cohort study of the temporal profile of viral load of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) from posterior oropharyngeal saliva samples and serum antibody responses, dated by symptom onset and correlated with clinical findings. Salivary viral load was highest during the first week after symptom onset and subsequently declined with time. EIA of IgG and IgM against internal viral nucleoprotein (NP) and surface spike protein receptor binding domain (RBD) showed correlation between antibody response and neutralising antibody titre.

### Implications of all the available evidence

Posterior oropharyngeal saliva specimens are non-invasive and acceptable to patients and can be used for initial diagnosis and subsequent viral load monitoring of COVID-19. The early peaking of viral load has important implications for transmission of SARS-CoV-2 in the community and hospital settings. EIA of IgG and IgM against internal viral NP and surface spike protein RBD can be used for those with delayed presentation or retrospective diagnosis of mild cases. As the positive EIA antibody level correlates well with neutralising antibody titre, further studies on its role in immunopathology or antiviral therapy are warranted.

done for SARS-CoV-2, although preliminary descriptive studies have been reported.[6–8]

In most studies of respiratory virus infections, serial sampling of nasopharyngeal or throat swabs is used for viral load monitoring. However, collection of nasopharyngeal or throat swab specimens can induce coughing and sneezing, which generates aerosol and is a potential health hazard for health-care workers. Collection of throat swabs also requires direct inspection of the patient's posterior pharynx and tonsils. Furthermore, collection of nasopharyngeal specimens is a relatively invasive procedure, which is uncomfortable and can even induce bleeding. A patient's reluctance to provide a sample can account for the paucity of timepoints in viral load studies of respiratory virus infections. Findings of previous studies have shown high concordance between saliva and nasopharyngeal aspirate as specimens for laboratory diagnosis of respiratory viruses.[9] We have reported use of posterior oropharyngeal (deep throat) saliva for diagnosis and viral load monitoring in a cohort of 12 patients with COVID-19.[6] Here, we report use of self-collected posterior oropharyngeal saliva samples from patients with COVID-19, which avoids close contact between health-care workers and patients, for viral load monitoring. We also monitored serial serum antibody levels of patients.

## Methods

### Patients

We included consecutive patients with laboratory confirmed COVID-19 who were admitted to Princess Margaret Hospital and Queen Mary Hospital in Hong Kong. In Hong Kong, patients were tested for SARS-CoV-2 based on clinical and epidemiological criteria as outlined and updated by the Hospital Authority. Initial laboratory confirmation was done using nasopharyngeal or sputum specimens at the Public Health Laboratory Centre of Hong Kong. We excluded patients if archived saliva or serum samples were insufficient for testing.

This study was approved by the Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster (UW 13-372). Since archived specimens were used, written informed consent was waived. 12 of 23 patients included in this study have been reported previously,[6] but their clinical information, viral load by single copy RNA-dependent RNA polymerase-helicase gene, antibody response, or viral genome analysis has not been reported before.

### Procedures

For viral load monitoring, all patients were asked to produce an early morning saliva sample from the posterior oropharynx (ie, coughed up by clearing the throat) before toothbrushing and breakfast, because nasopharyngeal secretions move posteriorly and bronchopulmonary secretions move by ciliary activity to the posterior oropharyngeal area while the patients are in a supine position during sleep. Patients were instructed and supervised by nurses. Viral transport medium was added to the saliva specimen. If patients were intubated, we obtained endotracheal aspirate instead of posterior oropharynx saliva.[6,9–11] Our initial experience showed that such saliva samples are promising in viral load monitoring in patients with COVID-19.[6] We also retrieved serum remnant from blood samples taken for routine biochemical testing, and refrigerated these samples at –20°C until antibody testing could be done.

We recorded clinical findings in a predesigned database, including a patient's history and physical examination and findings of haematological, biochemical, radiological, and microbiological investigations. We defined severe disease as the need for supplemental oxygen, admission to the intensive care unit (ICU), or death.

We did in-house reverse transcriptase quantitative PCR (RT-qPCR) targeting the SARS-CoV-2 RNA-dependent-RNA-polymerase-helicase gene region, as described (appendix p 1).[12] We did EIAs for SARS-CoV-2 nucleoprotein (NP) and spike protein receptor binding domain (RBD), as described but with modifications.[13] Recombinant NP and spike protein RBD of SARS-CoV-2 were used for the EIAs. We assessed the purity of NP and RBD by sodium dodecyl sulphate polyacrylamide gel electrophoresis and western blotting (figure 1A, B; appendix pp 2–3). A positive sample was included in each run as a positive control. We used an archived anonymous sample from 2018 as a negative control.[14] The cutoff for seropositivity was set as the mean value of 93 anonymous archived serum specimens from 2018, plus 3 SDs. We verified the validity of EIAs by competitive EIA (appendix p 6) and by western blotting, using patients' serum samples (figure 1C, D; appendix p 9). We did microneutralisation assays and virus culture, as described (appendix pp 1–5).[6,15] We did whole-genome sequencing using the Oxford Nanopore MinION device (Oxford Nanopore Technologies, Oxford, UK), as described (appendix p 4).[1]

### Statistical analysis

We did statistical analyses using SPSS version 26.0 or PRISM version 6.0. We compared categorical variables using Fisher's exact test and continuous variables with the Mann-Whitney *U* test. We used Spearman's correlation to assess the relation between age and viral load. A p value less than 0·05 was judged statistically significant.

### Role of the funding source

The funders had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all data in the study and had final responsibility for the decision to submit for publication.

## Results

Between Jan 22, 2020, and Feb 12, 2020, 30 patients were screened for inclusion, of whom 23 were included (13 male and ten female). Ten patients had severe COVID-19, of whom all required oxygen supplementation, and 13 patients had mild disease. The median age of patients was 62 years (range 37–75). 11 (48%) of 23 patients had chronic medical illnesses, and the most common underlying diseases were hypertension in six (26%) patients and diabetes in four (17%). Chronic comorbidities were more common among patients with severe COVID-19 (seven [70%] patients with severe disease had chronic comorbidities *vs* four [31%] with mild disease), although this difference was not significant (table). Five patients were admitted to the ICU, including three who required intubation. Two patients died.

The median interval between symptom onset and hospitalisation was 4 days (range 0–13). On presentation, the most common symptom was fever in 22 patients (96%), followed by cough in five (22%), chills in four (17%), and dyspnoea in four (17%; table). Dyspnoea was significantly more frequent among the ten patients with severe disease than among those with mild disease (four [40%] of ten *vs* none [0%] of 13; p=0·024). Serum alkaline phosphatase was significantly higher among patients with severe disease than among those with mild disease (74 U/L [range 56–149] *vs* 60 U/L [38–118]; p=0·026). The lymphocyte count was lower among patients with severe disease than among those with mild disease ($0·65 \times 10^9$ cells per L [range 0·30–1·90] *vs* 1·03 [0·57–2·25]), but this difference was not significant (p=0·088). Lymphopenia and neutrophilia were present in a higher proportion of patients with severe disease

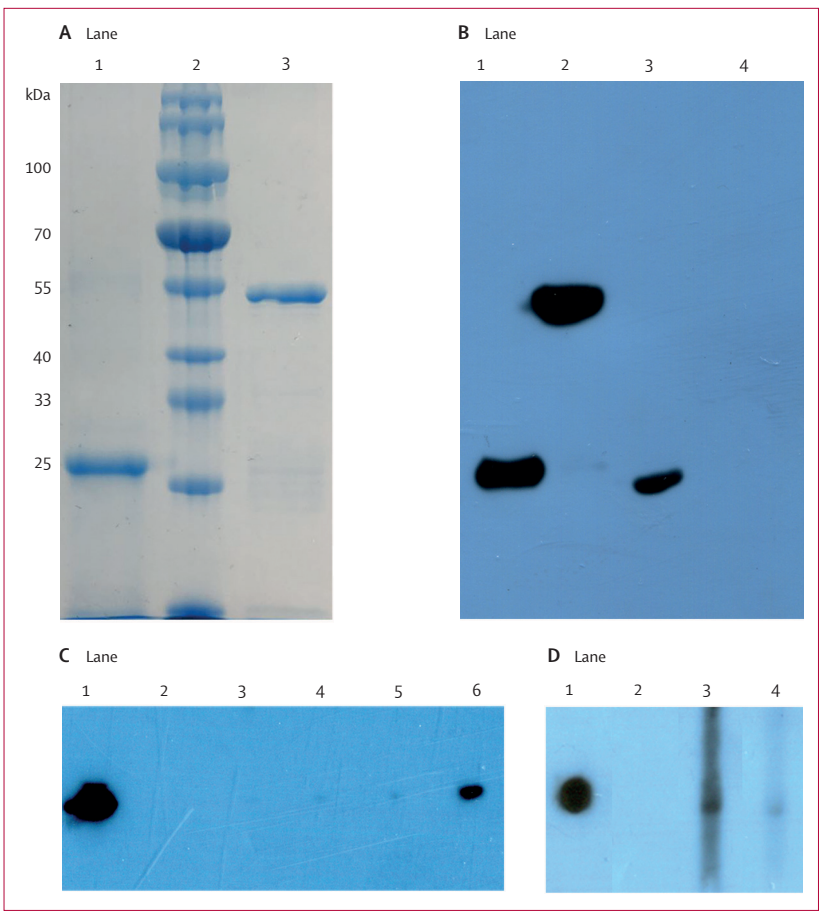


***Figure 1:*** **Recombinant NP and RBD of spike protein used for EIA**
(A) Sodium dodecyl sulphate-polyacrylamide gel electrophoresis showing purity of His-tagged RBD of spike protein (lane 1) and His-tagged NP (lane 3). Lane 2 is protein molecular weight marker. (B) Western-blot analysis of RBD of spike protein (lane 1) and NP (lane 2) using anti-His monoclonal antibody. Positive control (NP of severe fever with thrombocytopenia syndrome virus) is in lane 3 and negative control (GST-tagged protein) is in lane 4. (C) Western-blot confirmatory assay of NP using patient's serum. Anti-His monoclonal antibody in lane 1, negative patient serum in lane 2, serum samples from a patient with COVID-19 obtained during the acute phase of illness (5 days after symptom onset) in lane 3 (dilution 1 part to 100 parts) and during the convalescent phase (18 days after symptom onset) in lane 4 (dilution 1 part to 3200 parts), lane 5 (1 part to 1600 parts), and lane 6 (1 part to 800 parts). (D) Western-blot confirmatory assay with spike protein RBD using serum samples from patients with COVID-19. Anti-His monoclonal antibody in lane 1, negative patient serum in lane 2, serum from two patients with COVID-19 in lanes 3 and 4 (dilution 1 part to 100 parts). NP=nucleoprotein. RBD=receptor-binding domain. His=polyhistidine. GST=glutathione S-transferase. COVID-19=coronavirus disease 2019.

See Online for appendix

| | Severe disease (n=10) | Mild disease (n=13) | p value |
|---|---|---|---|
| Age, years | 66 (39–75) | 56 (37–75) | 0·10 |
| Sex | | | |
| Female | 4 (40%) | 6 (46%) | >0·99 |
| Male | 6 (60%) | 7 (54%) | ·· |
| Chronic comorbidities | | | |
| Hypertension | 4 (40%) | 2 (15%) | 0·34 |
| Chronic heart disease | 0 (0%) | 2 (15%) | 0·49 |
| Chronic lung disease | 1 (10%) | 0 (0%) | 0·44 |
| Chronic kidney disease | 1 (10%) | 0 (0%) | 0·44 |
| Diabetes | 2 (20%) | 2 (15%) | >0·99 |
| Gout | 2 (20%) | 0 (0%) | 0·18 |
| Hyperlipidaemia | 2 (20%) | 0 (0%) | 0·18 |
| None | 3 (30%) | 9 (69%) | 0·10 |
| Presenting symptoms | | | |
| Fever | 10 (100%) | 12 (92%) | >0·99 |
| Chills | 2 (20%) | 2 (15%) | >0·99 |
| Dyspnoea | 4 (40%) | 0 (0%) | 0·024 |
| Cough | 1 (10%) | 4 (31%) | 0·34 |
| Runny nose | 1 (10%) | 1 (8%) | >0·99 |
| Blocked nose | 0 (0%) | 1 (0%) | >0·99 |
| Sore throat | 1 (10%) | 0 (0%) | 0·44 |
| Chest discomfort | 1 (10%) | 0 (0%) | 0·44 |
| Nausea | 1 (10%) | 0 (0%) | 0·44 |
| Diarrhoea | 2 (20%) | 0 (0%) | 0·18 |
| Myalgia | 2 (20%) | 0 (0%) | 0·18 |
| Malaise | 2 (20%) | 1 (8%) | 0·56 |
| Duration of symptoms before admission, days | 4 (0–13) | 4 (0–7) | 0·41 |
| Blood tests on admission | | | |
| Haemoglobin, g/dL | 12·8 (11·6–14·5) | 13·5 (10·1–15·2) | 0·69 |
| Haemoglobin <13·7 g/dL (male) or <11·9 g/dL (female) | 4 (40%) | 6 (46%) | >0·99 |
| Total white blood cell count, $\times 10^9$ per L | 5·1 (2·4–10·4) | 4·9 (3·3–8·1) | 0·83 |
| Total white blood cells <3·7 $\times 10^9$ per L | 2 (20%) | 2 (15%) | >0·99 |
| Neutrophil count, $\times 10^9$ per L | 3·6 (1·3–9·5) | 3·8 (2·0–5·2) | 0·78 |
| Neutrophils >5·8 $\times 10^9$ per L | 3 (30%) | 0 (0%) | 0·068 |
| Lymphocyte count, $\times 10^9$ per L | 0·65 (0·30–1·90) | 1·03 (0·57–2·25) | 0·088 |
| Lymphocytes <1·0 $\times 10^9$ per L | 8 (80%) | 5 (38%) | 0·090 |
| Platelet count, $\times 10^9$ per L | 170 (92–313) | 182 (144–356) | 0·34 |
| Platelets <145 $\times 10^9$ per L | 4 (40%) | 1 (8%) | 0·13 |
| Sodium, mmol/L | 138 (128–142) | 139 (134–142) | 0·38 |
| Sodium <136 mmol/L | 4 (40%) | 1 (8%) | 0·13 |
| Potassium, mmol/L | 3·7 (3·1–5·3) | 3·8 (2·8–4·3) | 0·74 |
| Potassium <3·4 mmol/L | 2 (20%) | 2 (15%) | >0·99 |
| Urea, mmol/L | 3·9 (3·3–9·4) | 4·2 (2·1–9·9) | 0·74 |
| Urea >7·4 mmol/L | 2 (20%) | 1 (8%) | 0·56 |
| Creatinine, μmol/L | 76 (46–129) | 62 (54–126) | >0·99 |
| Creatinine >110 μmol/L | 1 (10%) | 1 (8%) | >0·99 |
| Alkaline phosphatase, U/L | 74 (56–149) | 60 (38–118) | 0·026 |
| Alkaline phosphatase >97 U/L | 2 (20%) | 1 (8%) | 0·56 |
| Alanine aminotransferase, U/L | 32 (16–88) | 26 (9–133) | 0·56 |
| Alanine aminotransferase >53 U/L | 1 (10%) | 3 (23%) | 0·60 |

(Table continues on next page)

than in those with mild disease, but the differences were not significant (p=0·090 and p=0·068, respectively).

Chest radiographic abnormalities were seen in 15 (65%) patients. Multifocal ground-glass lung opacities were seen in 17 (74%) patients on CT. SARS-CoV-2 RNA was detected in blood samples in five (22%) patients and rectal swabs in four (27%), but the detection rate between severe and mild cases did not differ (p=0·62 and p=0·57, respectively; table). SARS-CoV-2 RNA was not detected in any urine specimens. Lopinavir–ritonavir with or without ribavirin or interferon beta 1b was given in 18 (78%) patients at different timepoints after symptom onset.

In total, 173 respiratory specimens were obtained from 23 patients (mean 7·5 respiratory specimens per patient). The median viral load at presentation was 5·2 $\log_{10}$ copies per mL (IQR 4·1–7·0). SARS-CoV-2 RNA was not detected in the saliva of three (13%) patients. Specimens with undetectable viral load were assigned a value of 1 $\log_{10}$ copies per mL. No correlation was noted between days after symptom onset and initial viral load (Spearman's ρ=0·48; p=0·97). The viral load in posterior oropharyngeal saliva samples was highest during the first week of symptom onset then gradually declined (slope –0·15, 95% CI –0·19 to –0·11; $R^2$=0·71; figure 2). Endotracheal aspirate viral load was available from day 8 after symptom onset and showed a non-significant decline (slope –0·13, 95% CI –0·31 to 0·04; $R^2$=0·15). Of the 21 patients who survived, seven (33%) had viral RNA detected for 20 days or longer after symptom onset. No association was seen between prolonged detection of viral RNA (≥20 days after symptom onset) and severity of illness (p=0·35). One patient had viral RNA detected for up to 25 days after symptom onset; another patient had undetectable viral load on days 21 and 22 after symptom onset, with rebound of viral load on days 23 and 24, followed by 5 days of undetectable viral load.

A significant positive correlation between age and peak viral load was noted (Spearman's ρ=0·48, 95% CI 0·074–0·75; p=0·020; figure 3A). The median initial (p=0·56) and peak (p=0·52) viral loads in severe cases were about 1 $\log_{10}$ higher than those in mild cases, although the difference was not significant (figure 3B, C). The initial (p=0·49) and peak (p=0·29) viral loads did not differ between patients without comorbidities and those with comorbidities (figure 3D, E). For patients with both viral load and antibody results available in week 1 or week 3, median viral load was 6·70 $\log_{10}$ copies per mL (range 4·17–8·64), and the concomitant median optical density (OD) for anti-NP IgG was 0·13 (range 0·10–1·83) in week 1, whereas in week 3, median viral load was 4·91 $\log_{10}$ copies per mL (range 3·99–8·94) and the concomitant median OD for anti-NP IgG was 2·59 (range 2·12–2·65).

108 serum specimens were obtained from 23 patients (mean 4·7 serum specimens per patient). An increase was noted in IgG or IgM antibody levels against NP or RBD for most patients at 10 days or later after symptom

onset, as shown by OD values in EIA (figure 4). When comparing the onset of seropositivity between anti-RBD and anti-NP, more patients had earlier seropositivity for anti-RBD than anti-NP for both IgG (RBD earlier, ten [43%] of 23 *vs* NP earlier, two [9%] of 23) and IgM (RBD earlier, six [26%] of 23 *vs* NP earlier, four [17%] of 23). When comparing the onset of seropositivity between IgG and IgM, more patients had earlier seroconversion for IgG than IgM for anti-NP (IgG earlier, six [26%] of 23 *vs* IgM earlier, one [4%] of 23) and anti-RBD (IgG earlier, 13 [57%] of 23 *vs* IgM earlier, one [4%] of 23). For 16 patients with serum specimens available for 14 days or longer after symptom onset, the rate of seropositivity was 94% for anti-NP IgG (n=15), 88% for anti-NP IgM (n=14), 100% for anti-RBD IgG (n=16), and 94% for anti-RBD IgM (n=15).

To assess for host factors that affect the antibody titre, the correlation was analysed between the highest OD value during the convalescent period (third week after symptom onset) and age or comorbidities. Patients with comorbidities had a lower anti-RBD IgG OD than did those without comorbidities, although the difference was not significant (median OD, 0·65 *vs* 1·32; p=0·15; appendix p 7). No association was seen between comorbidity and anti-NP IgG or IgM OD values, or between age and anti-NP IgM or IgG or anti-RBD IgM or IgG OD values (appendix p 8).

Specimens with microneutralisation assay titres less than 10 were assigned a value of 5, and specimens with microneutralisation assay titres greater than 320 were assigned a value of 640. For one patient, a microneutralisation antibody assay was done with ten serial samples. The correlation between microneutralisation assay titres and anti-NP IgG ($R^2$=0·99) or anti-RBD IgG ($R^2$=0·96) was better than those between microneutralisation assay titres and anti-NP IgM ($R^2$=0·88) or anti-RBD IgM ($R^2$=0·87; figure 5).

Nanopore sequencing was successful for paired samples from four patients. The interval between the first and second specimens was 1–3 days. No viral mutations were identified between paired samples from individual patients.

## Discussion

We analysed the serial viral load, antibody kinetics, and viral genome of patients with COVID-19 in Hong Kong. For most patients, the viral load of SARS-CoV-2 was very high at presentation and declined steadily. Despite development of antibodies against surface and internal proteins of SARS-CoV-2, viral RNA could still be detected in posterior oropharyngeal (deep throat) saliva samples from a third of patients for 20 days or longer. Peak viral load correlated positively with age. Most patients had an antibody response at 10 days or later after onset of symptoms. Viral whole-genome sequencing of paired samples from four patients did not identify any single nucleotide polymorphisms.

| | Severe disease (n=10) | Mild disease (n=13) | p value |
|---|---|---|---|
| (Continued from previous page) | | | |
| Viral load in respiratory tract specimens | | | |
| Initial viral load, $\log_{10}$ copies per mL (IQR) | 6·17 (4·18–7·13) | 5·11 (3·91–7·56) | 0·56 |
| Peak viral load, $\log_{10}$ copies per mL (IQR) | 6·91 (4·27–7·40) | 5·29 (3·91–7·56) | 0·52 |
| Viral RNA detection | | | |
| ≥20 days in saliva* | 4 (50%) | 3 (23%) | 0·35 |
| Blood | 3 (30%) | 2 (15%) | 0·62 |
| Rectal swab† | 3 (38%) | 1 (14%) | 0·57 |
| Urine‡ | 0 (0%) | 0 (0%) | .. |

Data are n (%) or median (range), unless otherwise stated. For statistical analyses, the Mann-Whitney *U* test was done for continuous variables and Fisher's exact test was done for categorical variables. *For severe disease, the total number of patients was eight (two patients died <20 days after symptom onset). †For severe disease, samples were available for eight patients; for mild disease, samples were available for seven patients. ‡For severe and mild disease, samples were available for nine patients in each group.

***Table:*** **Patients' characteristics, by severity of disease**

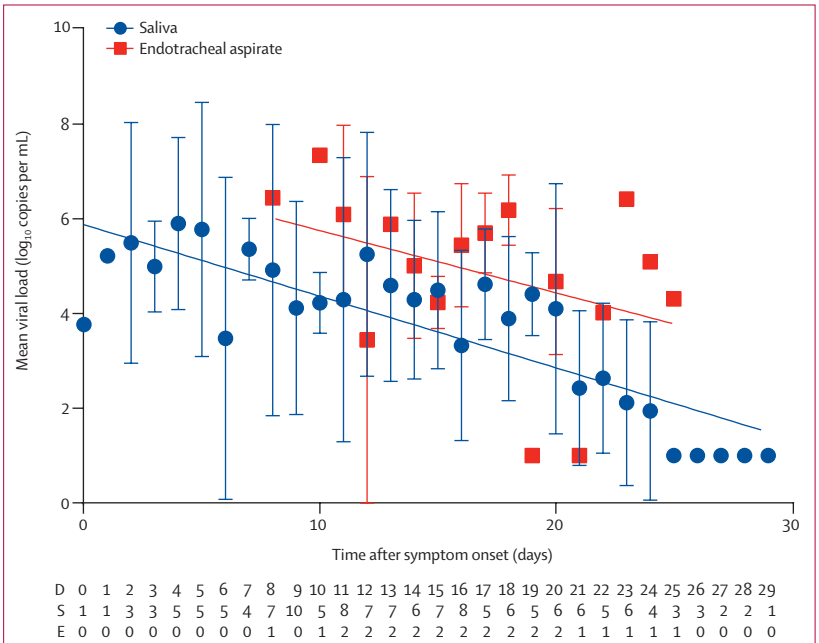


***Figure 2:*** **Temporal profile of serial viral load from all patients (n=23)**
Most viral load data are from posterior oropharyngeal saliva samples, except for three patients who were intubated, in whom viral load data from endotracheal aspirates are shown separately. Datapoints denote the mean; error bars indicate SD; slope represents best fit line. The number of patients who provided a sample on each day is shown in the table below the plot. D=days after symptom onset. S=saliva. E=endotracheal aspirate.

A high viral load on presentation of COVID-19 was recorded in our cohort, even for patients who were hospitalised shortly after symptom onset. Using nasal swab and throat swab, Zou and colleagues[8] have also reported a high viral load shortly after symptom onset. However, in that study, only cycle threshold values (not exact viral loads) were reported, and no statistical or correlative analysis was done with clinical variables such as age, comorbidities, disease severity, and antibody response. The viral load profile of SARS-CoV-2 is similar

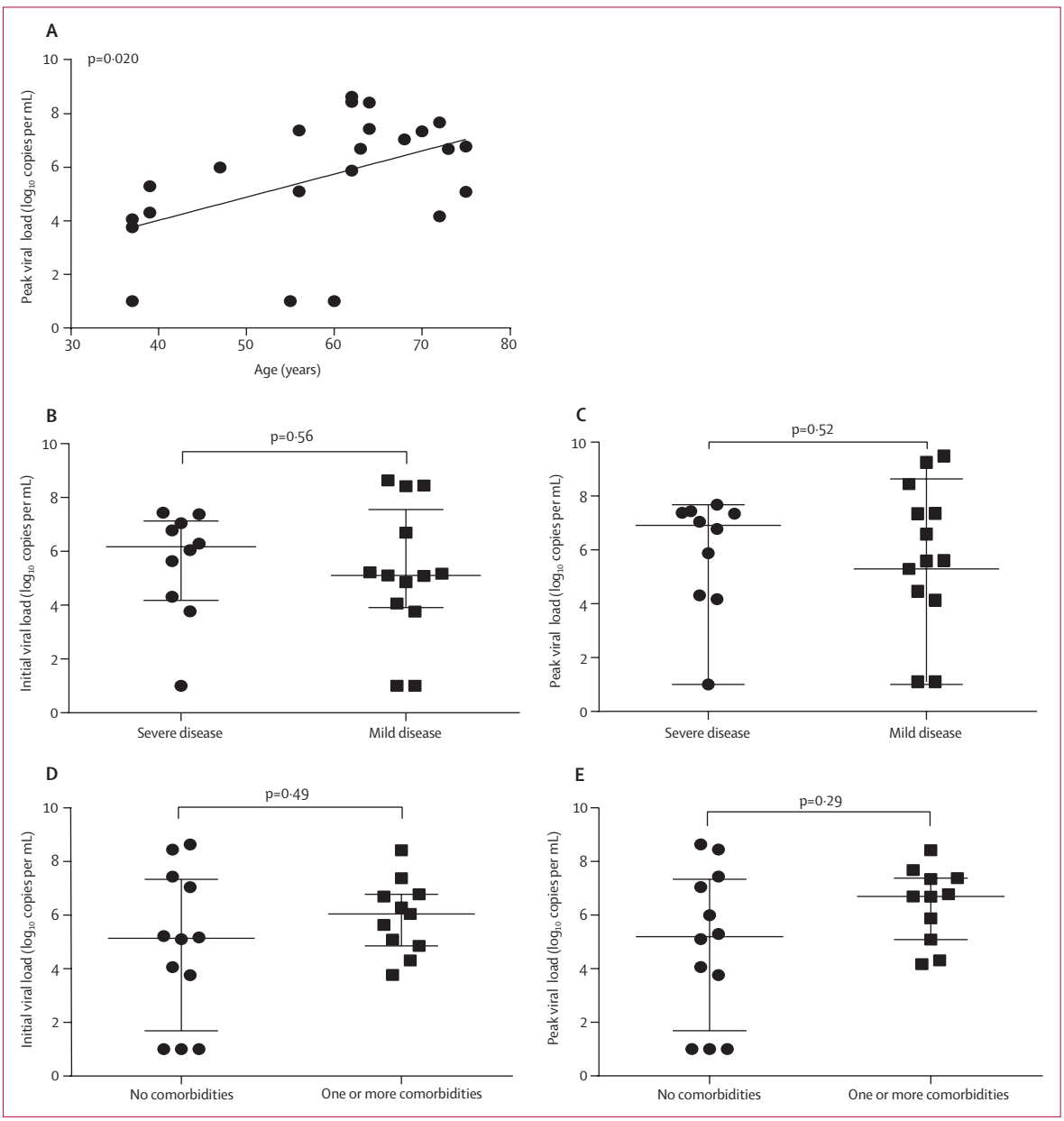


***Figure 3:*** **Relation between viral load and age or disease severity**
Correlation between age and peak viral load (A). Comparison of initial (B) and peak (C) viral load between severe and mild cases. Comparison of initial (D) and peak (E) viral load between patients with comorbidities and those without comorbidities.

to that of influenza, which peaks at around the time of symptom onset, but contrasts with that of SARS-CoV at around 10 days and that of MERS-CoV at the second week after symptom onset.[4,16,17] The high viral load on presentation suggests that SARS-CoV-2 can be transmitted easily, even when symptoms are relatively mild. This finding could account for the efficient person-to-person transmission noted in community and health-care settings. Clusters in families, workplaces, religious gatherings, and food premises have been widely reported.[18]

The viral load profile is important for guiding antiviral treatment. Since viral load had already peaked around the time of hospital admission, the risk of emergence of antiviral resistance could be similar to that of single-drug treatment of influenza by adamantanes, acid polymerase inhibitors, and neuraminidase inhibitors. However, our previous clinical trial of influenza treatment showed that a triple antiviral combination could significantly improve the clinical outcome and viral load profile and could reduce emergence of resistant virus quasi-species.[19] Currently, no standard treatment is available for

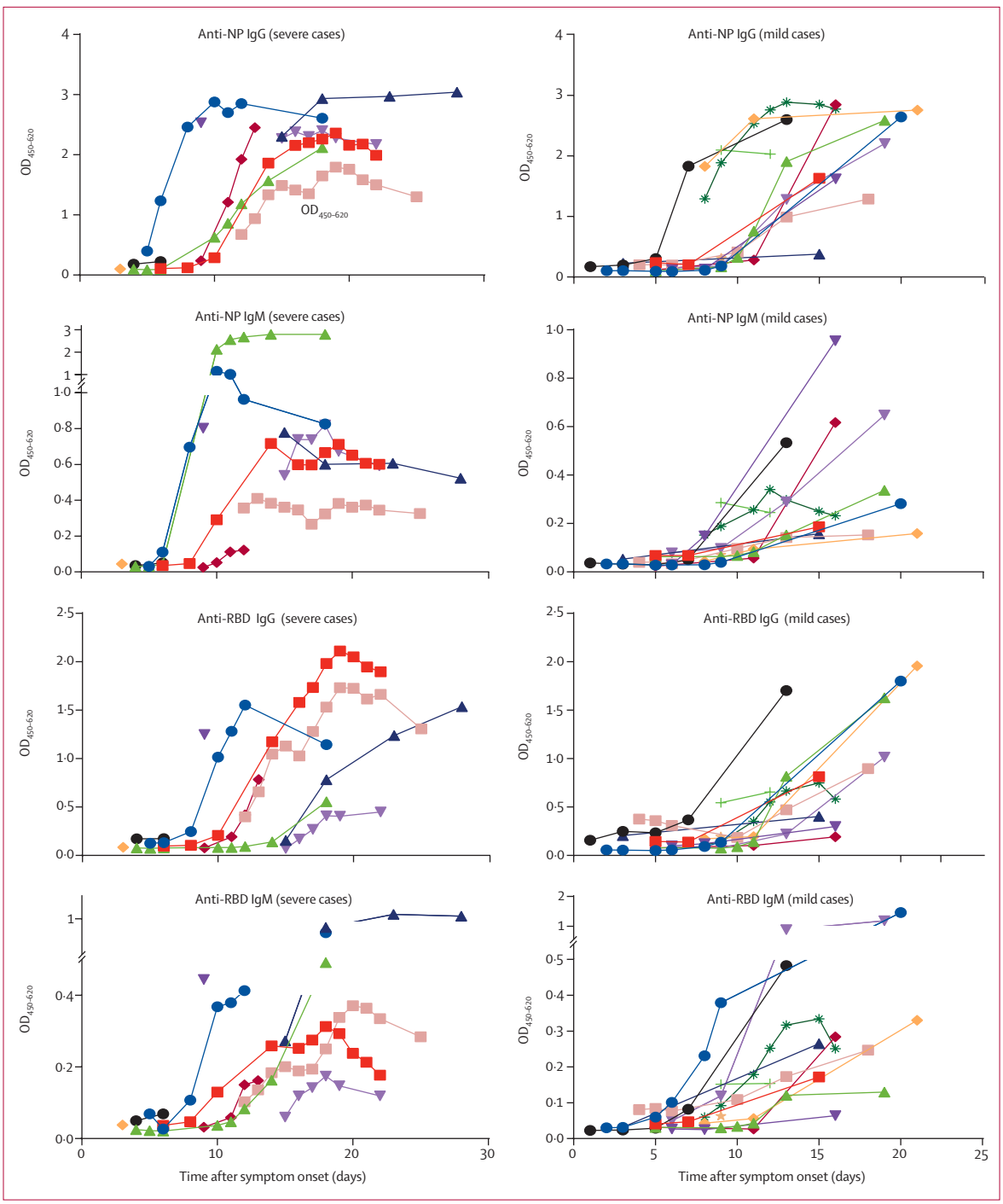


***Figure 4:*** **Temporal profiles of serum IgM and IgG against NP and spike protein RBD, as ascertained by EIA**
Each line represents an individual patient. NP=nucleoprotein. RBD=receptor-binding domain. $OD_{450-620}$=optical density at 450–620 nm.

COVID-19. For SARS-CoV infection, our previous treatment study showed that a combination of lopinavir–ritonavir and ribavirin led to significantly fewer complications (eg, acute respiratory distress syndrome) or deaths than reported with historical controls treated with ribavirin.[5] Lopinavir–ritonavir or interferon beta 1b also reduced lung damage and decreased viral load in a non-human primate model of MERS-CoV.[20] Lopinavir is a protease inhibitor with in-vitro activity against SARS-CoV and MERS-CoV. However, the idea that SARS-CoV 3C-like protease was the antiviral target of lopinavir was based purely on binding in computational modelling.[21]

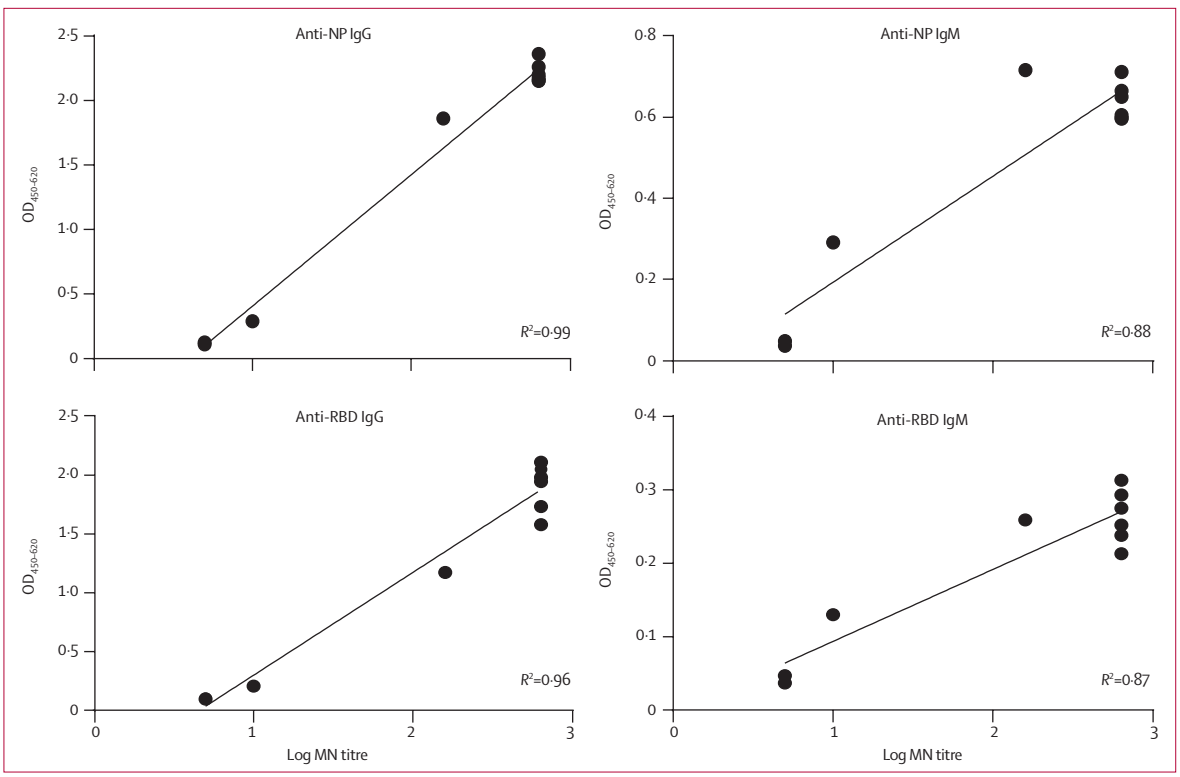


***Figure 5:*** **Correlation between MN antibody titres and anti-NP or anti-RBD IgG or IgM**
$OD_{450-620}$=optical density at 450–620 nm. MN=microneutralisation. NP=nucleoprotein. RBD=receptor-binding domain.

Other protease inhibitors and nucleotide analogues (eg, remdesivir [Gilead Sciences, Foster City, CA, USA]) are potential candidates for treatment. Combination treatment with virus-targeting and host-targeting agents to improve clinical outcome should be investigated.

Studies for SARS-CoV have shown that a high initial viral load was associated with death.[22] However, our study only showed that the median viral load was 1 $\log_{10}$ higher in severe cases than in mild cases, and the difference was not significant. But, older age was associated with a higher peak viral load. In a previous study of patients infected with SARS-CoV, older age was an independent factor associated with higher viral load,[23] as expected for immunosenescence, which impairs our innate and adaptive immune responses.

SARS-CoV-2 RNA could be detected for 20 days or longer in a third of patients who survived in our cohort, and one patient had SARS-CoV-2 RNA detected for 25 days. Prolonged detection of viral RNA of 20 days or longer was also commonly seen for patients with MERS-CoV or SARS-CoV infections.[4,16] Prolonged detection of viral RNA represents a challenge for the limited availability of hospital isolation facilities because patients might not be discharged until viral RNA is undetectable in respiratory specimens. Further studies are warranted to ascertain whether patients are shedding live virus, by viral culture of the prolonged RT-PCR-positive specimens obtained from patients with concomitant seropositivity when shedded virions are coated with host antibodies which render them non-infectious.

A criterion for discontinuation of transmission-based precautions is a negative RT-qPCR result from two sets of nasopharyngeal and throat swab specimens. In the current study, one patient with complete symptom resolution tested positive for SARS-CoV-2 again after 2 days of negative findings. Our results suggest that SARS-CoV-2 might be excreted at low levels despite clinical recovery. Thus, both serial viral load monitoring and antibody response should be considered when making decisions about infection control measures, because viral load seemed to be related inversely to serum antibody response in this study.

The antibody profile is vital for timing requests for serological assays and interpretation of antibody test results. Serological diagnosis is important for patients who present late with a very low viral load, below the detection limit of RT-PCR assays. Because most patients have rising antibody titres 10 days after symptom onset, collection of serial serum samples in the convalescent phase would be more useful. Serum IgG amounts can rise at the same time or earlier than those of IgM against SARS-CoV-2. By comparison with findings of a study on IgM and IgG EIA, in which more patients were seropositive for IgG than IgM at day 0 and day 5 of hospital admission,[24] a higher

proportion of patients in the current study also had earlier IgG than IgM seroconversion. However, this finding could also be accounted for by a lower sensitivity of the IgM EIA, which warrants investigations with more patients.

Serum antibody levels were not correlated with clinical severity. Notably, one patient with severe disease had an early antibody response 6 days after symptom onset. Deceased patients infected with SARS-CoV developed faster peak anti-spike antibody responses when compared with patients who recovered[25] and had subsequent reduced B-cell immunity with impaired neutralising ability. In a SARS-CoV macaque model, anti-spike IgG stimulated pulmonary proinflammatory responses and caused acute lung injury.[26] The detrimental effect of anti-spike IgG was attributable to the effect on wound-healing macrophages, which was mediated via the Fcγ receptor. Our findings showing correlation between antibody level detected by EIA and virus neutralisation titre are especially important for design of vaccine studies, and use of convalescent plasma or therapeutic monoclonal antibodies, which could improve clinical outcome or paradoxically cause immunopathological damage to the recipient.

Whole-genome sequencing on paired samples from four patients was successful and showed no differences in individually paired genome sequences. However, single nucleotide polymorphisms were shown to emerge during hospitalisation for MERS-CoV infection, using a targeted sequencing approach.[27] Further studies in more patients with samples obtained at longer intervals could be more informative.

A high viral load in throat wash and saliva (up to $10^8$ copies per mL of SARS-CoV RNA) was reported in 17 patients with SARS.[28] In a Chinese macaque model of SARS-CoV, salivary gland ducts were early targets of SARS-CoV and, therefore, were a likely source of the virions found in patients' saliva, particularly early in infection.[29] Because of these important findings, our study used posterior oropharyngeal saliva brought up by a throat-clearing manoeuvre to ascertain the temporal viral load profile. The posterior oropharynx is the meeting point between secretions coming from the posterior nasopharynx and the salivary glands and respiratory secretions swept up from the tracheal-bronchial tree. Testing of saliva could show viral shedding from both the salivary glands and the upper and lower respiratory tract. Moreover, because of greater patient acceptability for posterior oropharyngeal saliva samples than for nasopharyngeal or throat swabs, we obtained 7·5 respiratory specimens per patient for testing. Thus, our temporal viral load profile can be analysed by statistics, unlike previous clinical studies of viral kinetics of infections by highly pathogenic betacoronaviruses.[8,16] Further studies are needed to ascertain whether the salivary glands can be infected by SARS-CoV-2.

Our study has several limitations. First, we could only include a few patients, and viral load and antibody titre data were not available everyday. This limitation is a common problem in studies of emerging infections such as SARS-CoV and MERS-CoV.[16] The few patients enrolled does not allow for adjustment for potential confounding factors that could affect viral load or antibody response. Second, 48% of patients enrolled had chronic medical illness, which is a higher proportion than that reported in a large clinical series (24%).[30] Although a lower anti-RBD IgG level was noted among patients with comorbidities, further studies are warranted with more patients. Third, posterior oropharyngeal saliva samples cannot differentiate whether the virus is coming from the nasopharynx or from secretions from the lower respiratory tract; thus, our study cannot indicate whether SARS-CoV-2 has a predilection for both upper and lower respiratory tract. Moreover, some patients might not clear the throat effectively to cough out saliva from deep in the throat, which could decrease test sensitivity when compared with that of nasopharyngeal swabs, particularly in patients with predominant upper respiratory involvement or mild symptoms. Finally, the most abundantly expressed internal NP might have some cross-antigenicity between SARS-CoV-2 and SARS-CoV (90% amino acid homology) and, occasionally, OC43-CoV (38% amino acid homology).[31] Thus, the less abundantly expressed surface spike protein RBD, which is specific for SARS-CoV-2 and is the direct target for neutralising antibodies, was used to guard the specificity of our dual antibody assays.[32]

COVID-19 is an emerging infection with many unknowns. This study has shed light on viral kinetics and antibody response in patients and provides scientific evidence for guiding infection control policies and therapeutics. Further virological and immunological studies are needed to understand SARS-CoV-2 infection; infection control measures should be reviewed with the rapidly evolving epidemiology of COVID-19.

**Contributors**

KK-WT and K-YY contributed to study design, data collection, data analysis, data interpretation, the literature search, and writing of the report. OT-YT, W-SL, ART, T-CW, DCL, JM-CC, TS-HC, DP-LL, CY-CC, VC-CC, JF-WC, and IF-NH contributed to patients' recruitment, data collection, and clinical management. CC-YY, J-PC, L-LC, W-MC, K-HC, JDI, AC-KN, RW-SP, C-TL, ZC, and HC contributed to the experiments, data collection, data analysis, and data interpretation. All authors reviewed and approved the final version of the report.

**Declaration of interests**

We declare no competing interests.

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Chan Yin Chuen Memorial Charitable Foundation, Marina Man-Wai Lee, and the Hong Kong Hainan Commercial Association South China Microbiology Research Fund.

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## Supplementary appendix

This appendix formed part of the original submission and has been peer reviewed. We post it as supplied by the authors.

Supplement to: Kai-Wang To K, Tsang O T-Y, Leung W-S, et al. Temporal profiles of viral load in posterior oropharyngeal saliva samples and serum antibody responses during infection by SARS-CoV-2: an observational cohort study. *Lancet Infect Dis* 2020; published online March 23. https://doi.org/10.1016/S1473-3099(20)30196-1.

# APPENDIX

## SUPPLEMENTARY METHODS

### Nucleic acid extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) for SARS-CoV-2

Total nucleic acid (TNA) extraction was performed using NUCLISENS® easyMAG® nucleic acid extraction system (bioMérieux, Marcy l'Etoile, France). An in-house real-time RT-qPCR targeting the SARS-CoV-2 RNA-dependent-RNA-polymerase (RdRp)-helicase gene region was performed using QuantiNova Probe RT-PCR Kit (QIAGEN, Hilden, Germany) as we described previously [1]. The sequences of primers and probe are: Forward primer (5'-CGCATACAGTCTTRCAGGCT -3'); reverse primer (5'-GTGTGATGTTGAWATGACATGGTC -3'); probe (5' FAM-TTAAGATGTGGTGCTTGCATACGTAGAC -IABkFQ 3'). The reagent mixture (20 µL) contained 10 µl of 2x QuantiNova Probe RT-PCR Master Mix, 0.2 µl of QN Probe RT-Mix, 1.6 µl of each 10 µM forward and reverse primer, 0.4 µl of 10 µM probe, 1.2 µl of RNase-free water and 5 µl of TNA as the template. The thermal cycling condition was 45 °C for 10 min (reverse transcription), 95 °C for 5 min (PCR initial activation), followed by 45 cycles of 95 °C for 5 s and 55 °C for 30 s. All reactions were performed using the LightCycler® 480 II Real-Time PCR System (Roche, Basel, Switzerland).

### Cloning and purification of (His)6-tagged recombinant receptor binding domain (RBD) and nucleocapsid protein (NP) of SARS-CoV-2

The genes encoding the spike RBD (amino acid residues 306 to 543 of the spike protein) and full length NP of SARS-CoV-2 were codon-optimized, synthesized and cloned into the *NdeI*

site and *XhoI* site of expression vector pET-28b(+) (Novagen, Madison, WI, USA) in frame respective and upstream of the series of six histidine residues. The recombinant RBD and NP were expressed and purified using the $Ni2^{+}$-loaded HiTrap Chelating System (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions. The purity of RBD and NP was assessed by sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and western blotting (Figure 1). Nucleoprotein of severe fever with thrombocytopenia syndrome virus was used as a positive control [2]. The purified RBD and NP were separated electrophoretically in a 12% gel and transferred to a nitrocellulose membrane. After blocking with 3% BSA and 7% skim milk (Sigma-Aldrich), the membrane was incubated with anti-His monoclonal antibodies at a dilution of 1:4000 (Abcam, Cambridge, MA) for 1 hour at room temperature. After washing, the membrane was incubated with 1:4000 diluted goat anti-mouse horseradish peroxidase (Sigma-Aldrich) for 30 mins at room temperature, and developed by incubation with Amersham ECL Advance Western Blotting Detection Kit (GE Healthcare, Fairfield, CT). The concentration of purified RBD and NP were determined by using the Bicinchoninic Acid Protein Assay Kit (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions. Nucleoprotein of severe fever with thrombocytopenia syndrome virus was used as a positive control [2].

**Enzyme immunoassay (EIA) for NP and spike RBD**

Briefly, 96-well immunoplates (Nunc Immuno modules; Nunc, Denmark) were coated with 100 μl/well (0.1 μg/well) of SARS-CoV-2 NP or spike RBD in 0.05 M NaHCO3 (pH 9.6) overnight at 4°C and then followed by incubation with a blocking reagent. After blocking, 100 μL heat-inactivated serum samples at 1:100 dilution was added to the wells and incubated at room temperature for 1 h. For the determination of IgM, the serum was first mixed with an IgG

inactivation agent (Gullsorb) to inactivate serum IgG. The attached antibodies were detected using horseradish-peroxidase-conjugated goat anti-human IgG or IgM antibody (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). The reaction was developed by adding diluted 3,3',5,5'-tetramethylbenzidine single solution and stopped with 0.3 N $H_2SO_4$. The optical density (OD) was read at 450 nm. A single positive sample was included in each run as positive control. An archived anonymous sample from 2018 used in our previous study was used as negative control [3]. To determine the cutoff value for positivity, the mean value of 93 anonymous archived serum specimens from 2018 plus 3 standard deviations was used as the cutoff. The cutoff values are: anti-NP IgG, 0.523; anti-NP IgM, 0.177; anti-RBD IgG, 0.108; and anti-RBD IgM, 0.085. The original Western blot film of Figure 1D is shown in Supplementary Figure S4.

For competitive EIA, serially-diluted heat-inactivated serum samples (from 1:100 to 1:12800) were mixed with 10 μg SARS-CoV-2 NP or spike RBD, and incubated at 37°C for 1 h before adding to 96-well immunoplates as in the EIA assay described above. The OD~~450~~ of serum samples that were pre-mixed with NP or RBD was compared with those that were not pre-mixed.

**Microneutralization assay**

Microneutralization (MN) assay and virus culture were performed as we described previously [4,5]. Briefly, serum samples were serially diluted in 2-folds with minimum essential medium from 1:10 to 1:320. Diluted sera were mixed with 100 $TCID_{50}$ of SARS-CoV-2 and incubated at 37°C for 1 hour. The mixture was added to VeroE6 cells and incubated at 37°C and 5% $CO_2$. Cytopathic effect was determined by examination under inversion microscopy after 72

hours of incubation. The microneutralization antibody titer was the highest dilution with 50% inhibition of cytopathic effect.

**Whole genome sequencing and genome analysis by bioinformatics**

Whole genome sequencing was performed using the Oxford Nanopore MinION device (Oxford Nanopore Technologies) as we described previously [6]. Briefly, to deplete host cells, respiratory specimens were centrifuged at 16,000 × g for 2 min, and the supernatant was used for subsequent RNA extraction. RNA was extracted using QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany). DNase treatment (TURBO DNA-free Kit, Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) was used in removing residual host DNA. Whole genome amplification of SARS-CoV-2 was performed using sequence-independent single-primer amplification (SISPA) approach. Bioinformatics analysis was performed using an in-house pipeline. The consensus sequences and raw reads have been deposited into GenBank (Accession number: MT114412-19) and Bioproject (Acession number: PRJNA608242).

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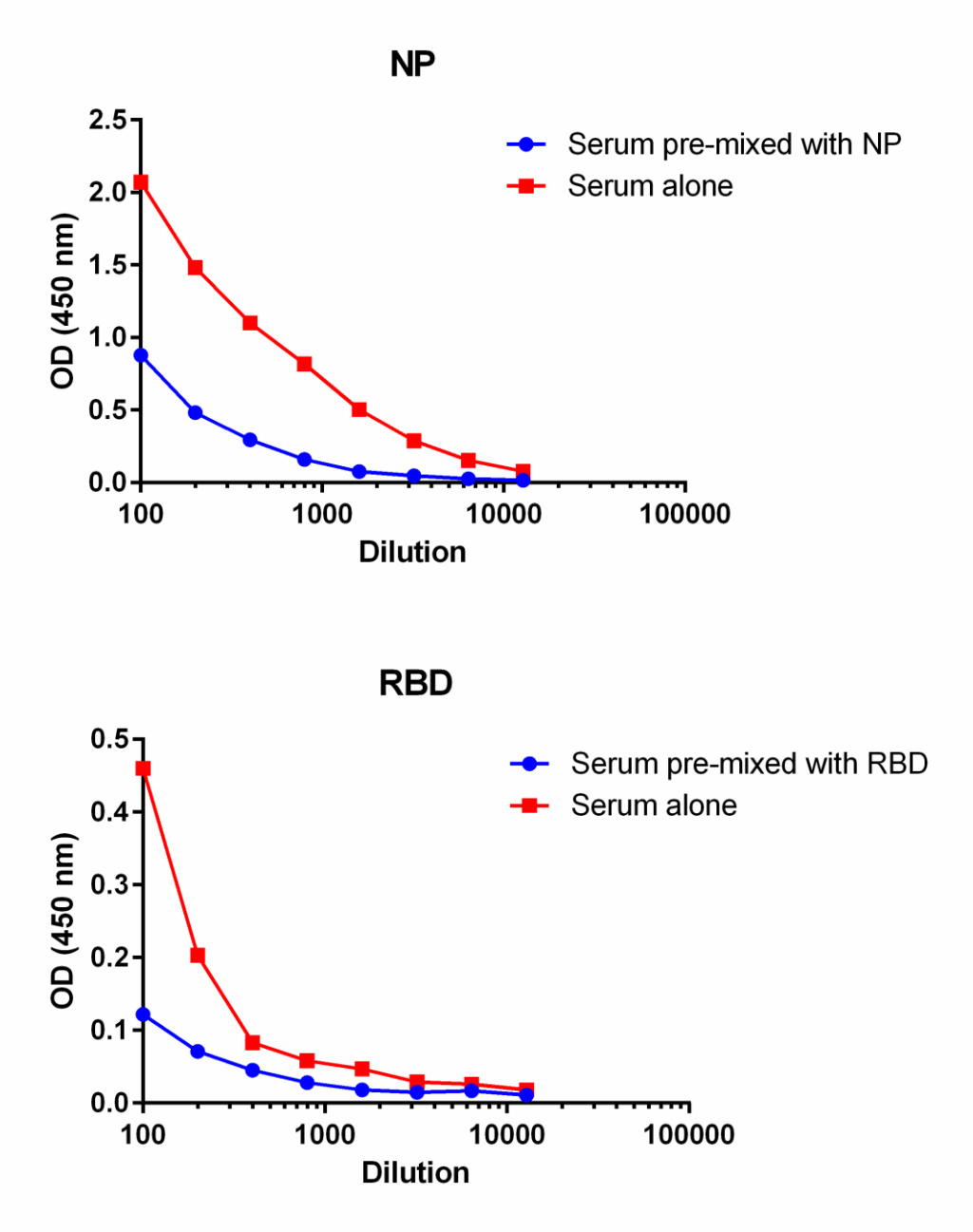
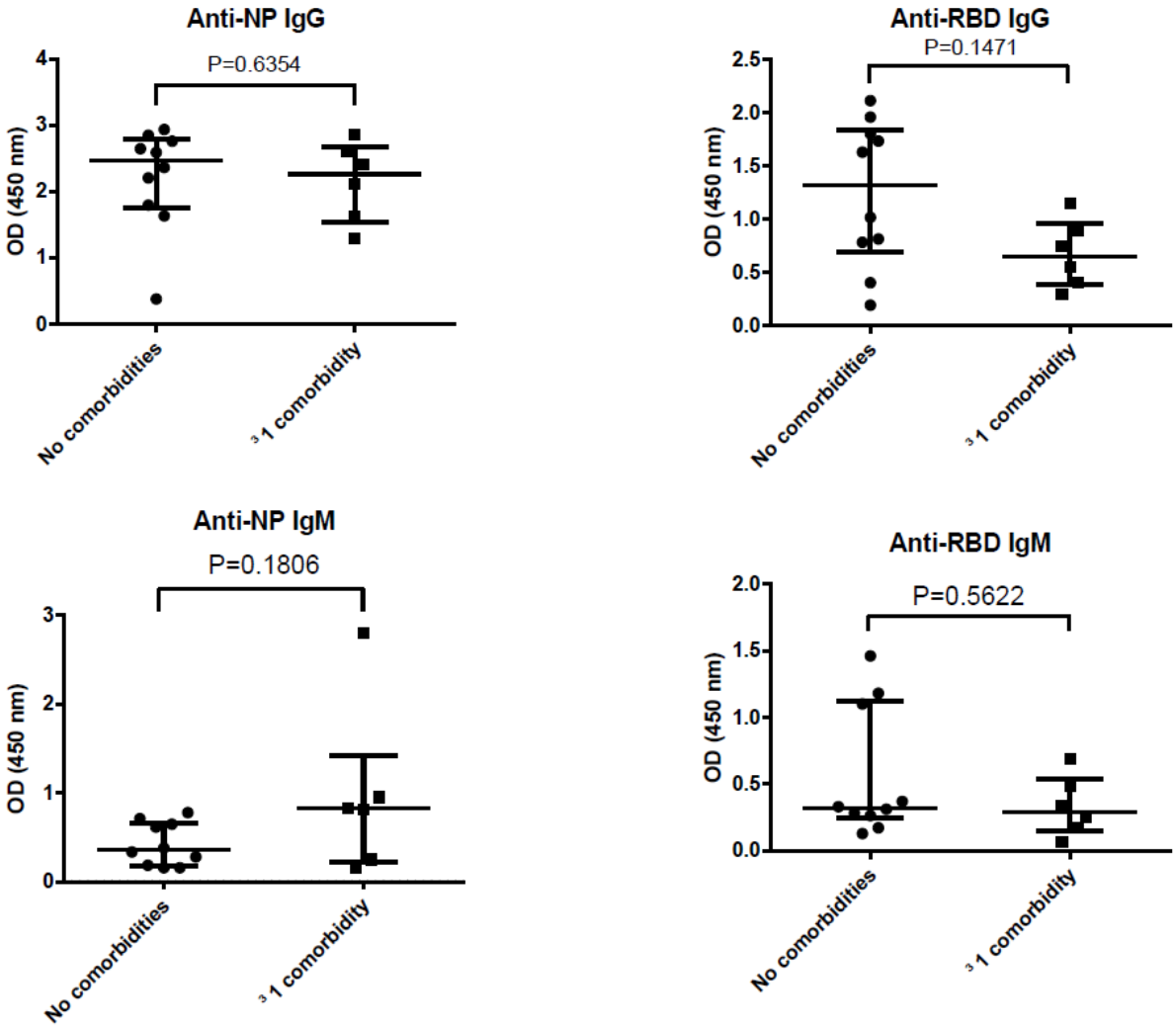
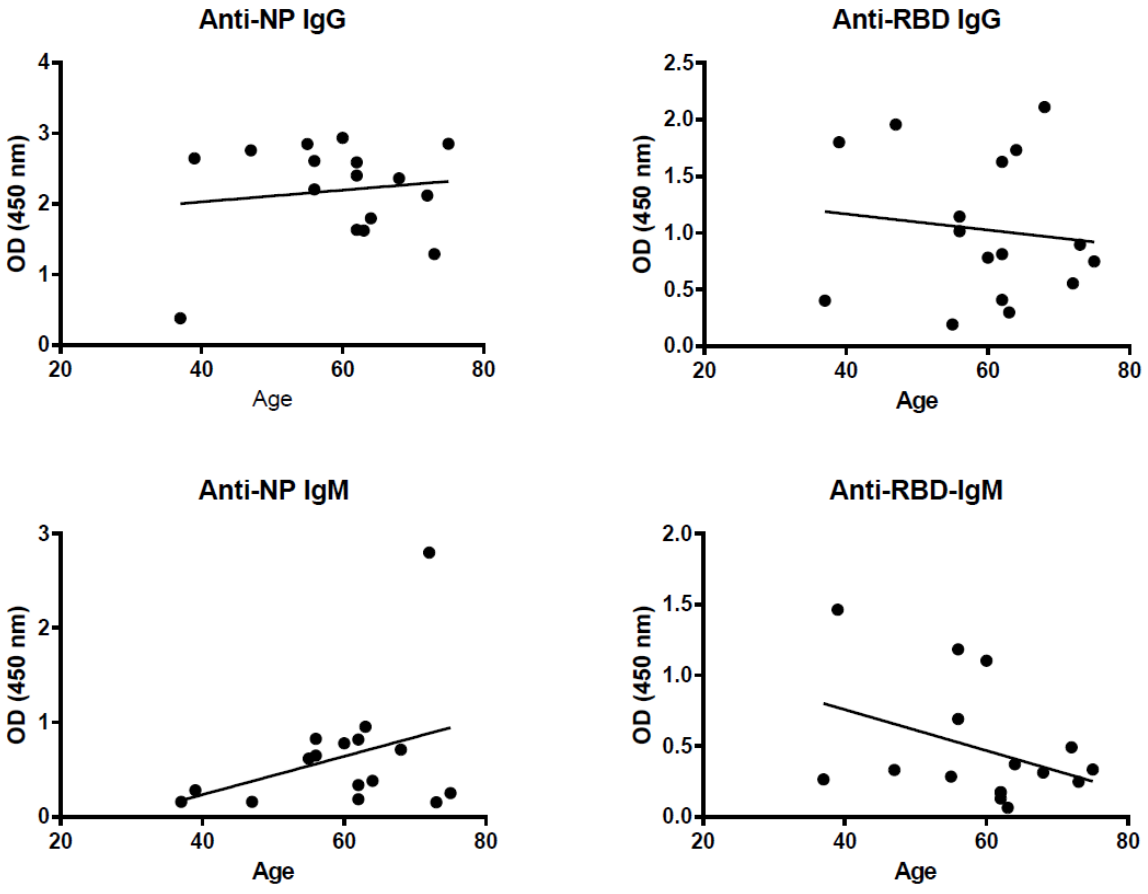
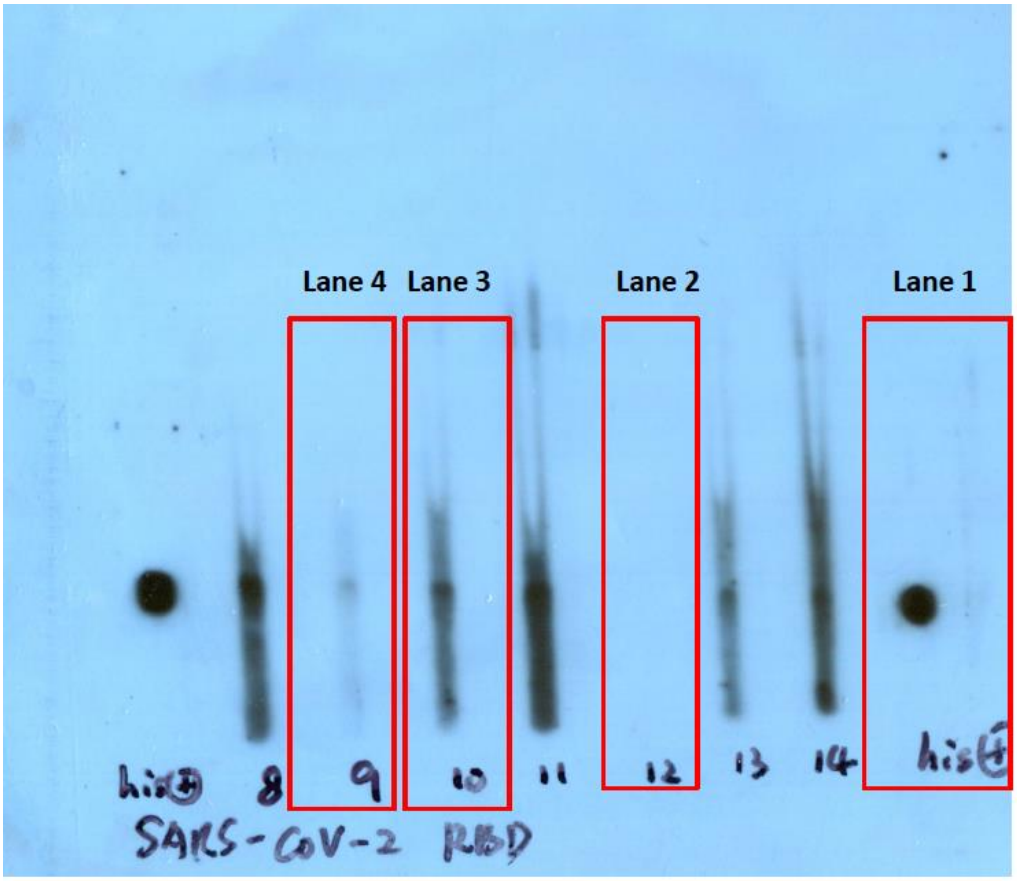
**Supplementary Figure S1. Competitive enzyme immunoassay for SARS-CoV-2 NP and RBD IgG**



**Supplementary Figure S2.** Comparison of antibody titers between patients with and without comorbidities



**Supplementary Figure S3.** Correlation between age and antibody titers.



**Supplementary Figure S4.** Original Western blot films of Figure 1D.