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Diagnostic Value of Nucleocapsid Protein in Blood for SARS-CoV-2 Infection

Running head: an emerging biomarker for COVID-19

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Non-standard abbreviations: N-Ag, nucleocapsid protein; EUA, emergency use authorization; RT-PCR, reverse-transcription real-time polymerase chain reaction; CRISPR, clustered regularly interspaced short palindromic repeats; POCT, point-of-care testing; S, spike protein; RBD, receptor binding domain; NP, nasopharyngeal; ICU, intensive care units; non-ICU, other departments in hospital; IQR, interquartile range; LOB, limit of background; Ct, cycle threshold; RFU, relative fluorescent units ROC; Receiver operating characteristic curves; AUC, area under the ROC Curve; CI, confidence interval; NIO, non-invasive oxygenation; MV, mechanical ventilation.

ABSTRACT

BACKGROUND: Biomarkers have been widely explored for COVID-19 diagnosis. Both viral RNA or antigens (Ag) in the respiratory system and antibodies (Ab) in blood are used to identify active infection, transmission risk, and immune response but have limitations. This study investigated the diagnostic utility of SARS-CoV-2 nucleocapsid protein (N-Ag) in serum.

METHODS: We retrospectively studied 208 randomly-selected cases with PCR-confirmed SARS-CoV-2 infection. N-Ag concentrations were measured in remnant serum samples, compared to PCR or Ab results, and correlated to electronic health records for clinical value evaluation.

RESULTS: Serum N-Ag was detected during active infection as early as day 2 from symptom onset with a sensitivity of 81.5%. Within one week of symptom onset, the sensitivity and specificity reached 90.9% (95% CI, 85.1–94.6%) and 98.3% (95% CI, 91.1–99.9%), respectively. Moreover, serum N-Ag concentration is closely correlated to disease severity, reflected by highest level of care, medical interventions, chest imaging, and the length of hospital stays. Longitudinal analysis revealed the simultaneous increase of Abs and decline of N-Ag.

CONCLUSIONS: Our study validated serum N-Ag as a biomarker for SARS-CoV-2 acute infection with high sensitivity and specificity compared to viral RNA in the respiratory system. We further revealed the correlation between serum N-Ag concentrations and disease severity and the inverse relationship of N-Ag and Abs. The diagnostic values of serum N-Ag, as well as technical and practical advantages it could offer, may meet unsatisfied diagnostic and prognostic needs during the pandemic.

Introduction

The coronavirus disease 2019 (COVID-19) pandemic caused by the infection of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has dramatically changed the world. In one year, greater than 110 million cases have been confirmed, with over 2.5 million deaths (*1*). Laboratory diagnostics play paramount roles in managing the ongoing pandemic, not limited to detection of active infection but also for the evaluation of transmission risk, immune response, disease severity, and prognosis. To develop and evaluate a diagnostic test, clinical significance, analytical performance, and practicality of implementation should all be taken into account.

Biomarkers for COVID-19, including viral RNA, proteins, and antibodies (Ab), have been widely explored and implemented into practice, which reveal different aspects and serve for different clinical indications (*2, 3*). By the end of 2020, the Food and Drug Administration (FDA) had approved more than 300 tests in the scope of emergency use authorization (EUA), including 203 molecular diagnostic tests plus 32 lab developed tests, 64 Ab tests, and 12 antigen (Ag) tests (*4*). Molecular tests were developed to detect viral RNA, which target different loci of SARS-CoV-2 genome. Most molecular tests rely on reverse-transcription real-time polymerase chain reaction (RT-PCR), while methods based on sequencing, mass spectrometry, or clustered regularly interspaced short palindromic repeats (CRISPR)-Cas technology have also been described (*4-9*). The molecular tests specifically detect viral RNA, but the correlation of viral load with disease severity remains unclear. Nearly 40% of patients with SARS-CoV-2 viral RNA detected have no obvious symptoms (*10*). Currently, no quantitative viral RNA tests have been

cleared by the FDA. Serology tests detect serum Abs, including immunoglobulin M (IgM), immunoglobulin G (IgG), or both, against viral proteins, mostly spike (S), receptor binding domain (RBD) of S, or nucleocapsid (N) proteins. These serology tests can evaluate immune response and detect prior infection or vaccination, which are useful for surveillance and epidemiologic studies (*11*). However, it has been demonstrated that the seroconversion for IgM and IgG usually occurs between 10~12 and 11~14 days after symptom onset, respectively; and these Abs remain detectable in most cases for months (*12-14*). Therefore, the Centers for Disease Control and Prevention (CDC) do not recommend serology tests solely to diagnose acute infection (*2*). Ag tests mainly focus on viral proteins (*15*). The 12 FDA-approved Ag tests detect either N protein or RBD of S protein in respiratory secretions. Although these Ag tests have reported specificity as high as 99.5% (95% CI, 98.1% to 99.9%), their sensitivity varies, averaging only ~ 56.2% (95% CI, 29.55 to 79.8%) compared to molecular tests (*16*).

As SARS-CoV-2 primarily affects the respiratory system, the current molecular and antigen tests mainly examine the virus in specimens collected from the respiratory system, such as nasopharyngeal (NP) or nasal swabs, sputum, saliva, or bronchoalveolar lavage (*3, 5*)**.** Viral RNA in the blood is detectable in less than 20% of COVID-19 cases *(17, 18*). Recent studies have demonstrated N-Ag in the blood as an emerging biomarker for SARS-CoV-2 infection (*19-21*). Here, we investigate the diagnostic value of serum N-Ag for COVID-19 by evaluating its sensitivity and specificity for active infection, its correlation with disease severity, and the kinetics of N-Ag and Abs during disease progress.

Materials and Methods

Subjects and specimens

This study utilized a random sampling of remnant serum or plasma samples from routine clinical laboratory testing at Zuckerberg San Francisco General Hospital from March to July, 2020. All patients were RT-PCR positive for SARS-CoV-2 from NP swab testing. We identified 208 cases with a serum sample collected within 24 hours of swab collection, and 203 cases had enough remaining serum after routine clinical laboratory testing for further testing. The patients were 67% male, 75% Hispanic, with a median age of 48 years. 91 patients (44%) were hospitalized and 117 patients (56%) were outpatients. Of the hospitalized patients, 37 (41%) were hospitalized to the intensive care unit (ICU), 25 (27%) received mechanical ventilation, and 7 died.

To study the kinetics of N-Ag and Abs over time, a cohort of remnant serial samples from 16 patients in the ICU and 4 in other departments (non-ICU) were investigated. For each patient, ≥7 serum samples were collected for a period ≥ 7 days.

Clinical data extracted from electronic health records included demographic information, patient-reported date of symptom onset, symptoms, major comorbidities, highest level of care (asymptomatic, symptomatic but discharged to home, hospitalized to non-ICU, and hospitalized to ICU), medical interventions (non-invasive oxygenation, mechanical ventilation), chest imaging by X-ray or computed tomography (CT) (infiltrates, ground glass opacities, consolidation, and other pulmonary findings, or clear lungs), and length of hospital stay.

This study was approved by the institutional review board (IRB) of the University of California, San Francisco (IRB number 20-30387).

Serum N-Ag measurement and method validation

Serum N-Ag was detected by SARS-CoV-2 antigen quantitative assay kit (Biohit Healthcare) (20). Testing personnel were blinded to the clinical information. Serum samples (50 μl) and biotin-labeled anti-SARS-CoV-2 N protein Ab were sequentially added to a microplate precoated with mouse anti-SARS-CoV-2 N protein monoclonal Ab. If a sample contained N protein, a complex of [solid-phase Ab]-[N-Ag]-[biotin-labelled Ab] was formed. After plate washing with phosphate buffer solution five times, streptavidin labeled with horseradish peroxidase was added to form an immune complex through streptavidin-to-biotin binding. The unbound substances were washed away, and a substrate solution containing 3,3',5,5'-tetramethylbenzidine and urea hydrogen peroxide was added to the microplate. The reaction was stopped by a sulfuric acid solution, and absorbance values were measured by a multilabel plate reader PerkinElmer Victor X4 at 450 nm with 650 nm as a reference wavelength. The concentration of N-Ag in serum samples was calculated using a calibration curve of SARS-CoV-2 N calibrators (0, 5, 10, 40, and 160 pg/mL) measured in parallel. The limit of blank (LOB), of detection, and of quantitation were calculated to be 1.08, 1.66, and 2.89 pg/mL, respectively, with a linear range from 2.89 to 180.01 pg/mL. The cut-off was determined by the manufacturer as 2.97 pg/mL. For a sample with N-Ag concentration beyond the linear range, it was diluted 10X each time until the dilution fell into the linear range, and the concentration was calculated by the final concentration multiplied by the dilution factor. Any N-Ag

concentration under 1 pg/mL, lower than LOB, was transferred to 1 pg/mL for data representation on figures with logarithmic scale.

Serum Ab measurement

Serum Abs , including IgM and IgG against recombinant RBD of S and N proteins of SARS-CoV-2, were measured on the Pylon 3D automated immunoassay system (ET Healthcare) as previously described (*14*). The background-corrected signal was reported as relative fluorescent units (RFU), which was proportional to the concentrations of specific Abs in serum samples.

RT-PCR tests

All remnant serum samples were saved from individuals who were RT-PCR positive using methods that were FDA-approved for EUA, and all RT-PCR testing was performed in a CLIA-certified clinical laboratory. For the comparison of serum N-Ag concentration to viral RNA load in NP swabs, cycle threshold (Ct) values of RT-PCR were collected. Considering the reported Ct variation among different RT-PCR platforms (*22*), we checked Ct values obtained from Abbott M2000 only.

Amino-acid sequencing alignment of N proteins

The full-length sequences of N proteins in coronavirus, including SARS-CoV-2 (YP_009724397.2), SARS-CoV (YP_009825061.1), MERS-CoV (YP_009047211.1), 229E (APT69891.1), OC43 (QDH43730.1), HKU1(QHB49085.1) and NL63 (ABI20791.1), were submitted onto Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) and analyzed by Clustal2.1. to obtain the phylogenetic tree of N proteins and protein identity within the Coronavirus family. The sequence alignment map was drawn with SnapGene.

Statistical Analysis

Data analysis were performed with Prism 9. Mann-Whitney or Kruskal-Wallis tests were used to compare N-Ag concentrations in two groups or more, respectively. All data were considered as non-gaussian distribution, and P value were calculated with two-tailed hypothesis. Serum N-Ag concentrations were shown as median [25–75% interquartile range (IQR)].

Results

Kinetics of serum N-Ag in COVID-19 cases

To determine the kinetics of serum N-Ag during active infection, serum N-Ag results were grouped by days post symptom onset (Figure 1A and Table 1). The median [IQR] serum N-Ag concentration increased from 1 [1–167] pg/mL on day 0 (n=26), to 18 [1–211] pg/mL on day 1 (n=22), 116 [30–2015] pg/mL on day 2 (n=22), reached a peak during day 3–7 with the median value > 1000 [628–6466] pg/mL (n=99), and decreased thereafter to 437 [31–3416] pg/mL from day 8–14 (n=27) and 144 [1-13694]pg/mL from day 15–21 (n=7). No statistical differences were observed in the serum N-Ag concentration between day 3–7. Therefore, day 3–7 from symptom onset was considered as a peak time window with elevated and relatively stable serum N-Ag concentration. With the manufacturer-recommended cut-off at 2.97 pg/mL, the sensitivity increased dramatically from 42.3%, 68.2%, 86.4%, to 96.0% in the first four days following symptom onset, maintained at > 95.0% during day 3–7 and decreased thereafter.

The sensitivity and specificity of serum N-Ag

Using RT-PCR-based viral RNA detection as the reference, we evaluated the accuracy of serum N-Ag, which were collected within 24-hours of RT-PCR. Area under the receiver operating characteristic (ROC) curves were 0.961, 0.925, and 0.782 for samples collected within Day 1–7, Day 8–14, and Day 15–21 from symptom onset respectively (Figure 1B). Within 7 days from symptom onset, 130 out of 143 PCR-positive cases were positive for serum N-Ag, and 59 out of 60 PCR-negative cases were negative for serum N-Ag. Compared to RT-PCR results for COVID-19 diagnosis, the serum N-Ag test during day

1–7 from symptom onset yielded a sensitivity of 90.9% (95% CI:85.1–94.6%) and a specificity of 98.3% (95% CI: 91.1–99.9%) for SARS-CoV-2 infection (Table 2).

Potential cross-reactivity in cases with serum specimens from other respiratory viral infections, including human rhinovirus/enterovirus, metapneumovirus, respiratory syncytial virus, parainfluenza type 1 virus, and adenovirus was evaluated. No N-Ag signals were observed in these cases (n=16) (Figure S1A).

To further evaluate any cross-reactivity potential with other coronaviruses, we analyzed the amino acid sequence similarity of N proteins within the coronavirus family, including seasonal coronaviruses (229E, NL63, OC43, and HKU1) and SARS-CoV and middle east respiratory syndrome (MERS)-CoV. Compared to SARS-CoV-2, the N protein sequence identity is 89.7% for SARS-CoV, 48.6% for MERS-CoV, and 26.7–35.8% for coronaviruses 229E, NL63, OC43, and HKU1 (Figure S1B). SARS-CoV-2 N Ag was not detected in serum samples from individuals infected with 229E, OC43, or HKU1 (n=5) (Figure S1A). No serum samples with NL63, SARS-CoV, MERS-CoV infection were available for analysis.

The correlation of serum N-Ag concentration and disease severity

Next, we investigated whether there was any correlation between serum N-Ag concentration and disease severity, reflected by highest level of care, medical interventions, chest imaging, and length of hospital stay. To ensure N-Ag results were comparable, we selected all samples (n=99) which were collected during the peak period of N-Ag kinetics (day 3–7 from symptom onset) for disease severity correlation studies.

Among symptomatic cases (n=99), N Ag concentrations were significantly increased with highest level of care. Compared to asymptomatic patients (n=19) with serum N-Ag at 1 [1–121] pg/mL, the median [IQR] serum N-Ag concentration from day 3–7 was 1015 [31–3650] pg/mL in patients who were symptomatic, but discharged to home (n=52, P =0.0007), 3854 [912–5566] pg/mL in patients who were hospitalized to non-ICU (n=24, P<0.0001), and 10,712 [2697–17431] pg/mL in patients hospitalized to ICU (n=23, P<0.0001) (Figure 2A).

Serum N-Ag concentrations were significantly higher in groups receiving medical interventions for COVID-19: 1038 [29–4101] pg/mL in the control group without interventions (n=51), 3575 [1049–6443] pg/mL in cases with non-invasive oxygenation (n=32), and 12,041 [2901–19167] pg/mL in cases with mechanical ventilation (n=16) (Figure 2B).

96 out of the 99 cases were checked with either chest X-ray or CT to examine pulmonary injury. Among them, 86 cases had abnormal imaging reported, including infiltration, ground glass opacities, and/or consolidation. The median [IQR] N-Ag concentration increased from 224 [9–1138] pg/mL in cases with clear lungs (n=10) to 3098 [805–8012] pg/mL in cases with abnormal imaging (P=0.0005) (Figure 2C).

We further checked the correlation of serum-N Ag concentrations at admission with length of hospital stay (n=99). Depending on length of hospital stay, the 99 cases were divided into four groups, 0 days (n=40), 1–10 days (n=42), 11–20 days (n=6), and > 20 days (n=11). The median [IQR] concentration of serum N-Ag were 886 [29–4021] pg/mL, 2367 [791–6397] pg/mL, 11,225 [2547–17141] pg/mL, and 13,370 [5308–21241]

pg/mL, respectively (Figure 2D). The elevation of serum N-Ag was statistically significant when comparing the 11–20 days *vs.* 0 days (P=0.0207), >20 days *vs.* 0 days (P=0.0002), and >20 days *vs.* 1–10 days (P=0.030).

Comparing serum N-Ag level and viral load in swabs

To check whether there was any correlation between serum N-Ag level and viral load in the respiratory system, we compared serum N-Ag concentrations with Ct values of RT-PCR, which are inversely proportional to the logarithm of viral RNA in swabs. 102 out of 208 cases had both serum N-Ag concentrations and Ct values available, from which serum and swab samples were collected within 24 hours. No obvious correlation between serum N-Ag concentrations and Ct values was observed (Figure S2A).

Additionally, we investigated whether Ct values of RT-PCR correlated with disease severity. First, we checked the kinetics of Ct values by days from symptom onset and did not find dramatical change or trend within one week after symptom onset (Figure S2B). Therefore, Ct values obtained within the first week of symptom onset (n=84) were selected for disease-severity-correlation study. Similarly, we compared Ct values in relation to highest level of care, medical interventions, chest imaging, and across different length of stay. Patients hospitalized to the ICU, receiving mechanical ventilation, or requiring an extended hospital stay might have slightly lower Ct values, *i.e.* higher viral load. However, the trend was not statistically significant (Figure S2 C–F).

Inverse correlation of serum N-antigen and antibodies

The kinetics of serum N-Ag, IgG, and IgM over time were available for 20 cases (Figure 3A). During hospitalization, all cases, including 16 ICU cases and 4 non-ICU cases, started with high serum N-Ag concentrations although concentrations varied significantly between cases. The N-Ag concentration declined as serum IgM and IgG increased. The negative correlation of N-Ag with IgM and IgG in each case indicates an inverse relationship between N-Ag and Abs (Figure 3B and 3C).

Discussion

In this study, we evaluated the diagnostic value of serum N-Ag for COVID-19. This serum N-Ag test has a sensitivity of 90.9% (95% CI:85.1–94.6%) and a specificity of 98.3% (95% CI: 91.1–99.9%) for cases within day 1–7 of symptom onset compared to RT-PCR-based viral RNA testing using NP swabs. The test sensitivity reached 81.5% as early as day 2, meeting the minimum FDA requirement (≥ 80%) under EUA (*23*). The SARS-CoV-2 N-Ag assay does not appear to have cross-reactivity with other common respiratory viruses, including seasonal coronaviruses. More importantly, our study revealed a close positive correlation of serum N-Ag concentration to disease severity. The serum N-Ag concentrations significantly increased along the continuum of highest level of care from asymptomatic cases through those hospitalized to the ICU. Similarly, we observed significantly higher serum N-Ag concentrations in cases that required mechanical ventilation/oxygenation *vs*. those without and in cases with abnormal chest imaging *vs.* those with clear lungs. Of note, the serum N-Ag concentrations assessed in the peak window of antigenemia (day 3–7 from symptom onset) correlated with the length of hospital stay. We also found the kinetics of N-Ag and Abs in 20 patients revealed a

synchronous decrease in N-Ag and increase of IgG and IgM, suggesting immune response and Ag clearance. Therefore, serum N-Ag is a biomarker for SARS-CoV-2 infection with high sensitivity and specificity comparable to RT-PCR-based viral RNA tests, is closely correlated with disease severity, and demonstrates an inverse relationship with Ab development.

Compared to viral RNA or Ag in the respiratory system and Abs in the blood, serum N-Ag may offer extra diagnostic values and bridge a gap in clinical needs. The severity of COVID-19 varies dramatically from asymptomatic to critical illness or even death, and the uncertainty causes public panic and healthcare system overburden. Although molecular tests are well accepted as the gold standard, the correlation of viral RNA in swabs with disease severity remains debated and not significant as our data revealed. Nearly 40% of patients with detectable viral RNA have no apparent symptoms (*10, 24*). Remarkable variations of swab RNA tests were observed, which may attribute to intrinsic factors, such as uneven viral distribution in the respiratory system, inadequate sampling, RNA instability, and analytical method variations (*25*). Furthermore, viral RNA can be detected by RT-PCR for weeks after recovery, which may cause unnecessary isolation precautions or treatment (*26*). As an alternative to viral RNA, our data show serum N-Ag is highly sensitive in a peak window of detection associated with acute infection and quickly decreases after Ab development. Furthermore, peak levels of antigenemia correlate with disease severity in our data, suggesting a potential role of serum N-Ag for the prognosis of COVID-19.

In addition, serum N-Ag detected by immunoassays offers technical and practical advantages over molecular testing. The serum N-Ag test could be implemented on

automated platforms in clinical laboratories, offering large volume testing with rapid results using minimal labor. The method could also be transformed into point-of-care testing (POCT) with immunochromatographic lateral flow assays and blood sampling in the form of finger stick at home. Such POCT could meet the high-scale and time-sensitive requirements to help control the pandemic (*27*). Additionally, blood specimens may be lower risky to handle than respiratory specimens considering intact virus is less frequently detected in the blood (*18*). Serum N-Ag test could also minimize the analytical variation of RT-PCR tests in swabs, primarily due to sample collection and RNA instability. A panel of serum N-Ag and Abs only needs one blood specimen with simultaneous sample processing but can monitor COVID-19 infection from aspects, including active infection, convalescence, and immune response.

To date, three other groups have reported serum N-Ag as a potential biomarker with varied sensitivity and specificity for SARS-CoV-2 infection and provided limited correlation to disease severity (*19*-*21*). With enzyme-linked immunosorbent assay, Hingrat, *et al*. estimated the sensitivity of serum N-Ag as 79.3% (95% CI, 74.0–84.6%) for all cases or 93.0% (95% CI, 88.7–97.2%) for cases within 14 days from symptom onset (*19*); and Li, *et al.* reported a sensitivity of 92% (95% CI, 81.2–96.9%) (20). With a single-molecule array, Ogata, *et al.* measured SARS-CoV-2 proteins in blood samples collected within 10 days of PCR tests—without time information from symptom onset—and detected elevated N-Ag in 41 out of 64 (~64.1%) samples (*21*). One possible reason for the sensitivity variation from different reports is the time of sample collection relative to the disease course. As our kinetics study indicated, serum N-Ag increased quickly in the first few days after symptom onset, peaked around day 3–7, and declined in week

2~3. Therefore, the sensitivity varied over time dramatically. To detect acute infection, it is highly recommended to assess serum N-Ag within 1~2 weeks after symptom onset, for which period a sensitivity >90% has been reported by different groups. The delay from swab- to serum- collection may also contribute to the variation. In this study, collection of both specimens within 24 hours allowed for a direct comparison between serum N-Ag and viral RNA in swabs. Similarly, when evaluating the correlation of serum N-Ag with disease severity, it is important to ensure samples are selected from the same timeframe. Ogata, *et al.* demonstrated a higher concentration of serum N-Ag in patients admitted to ICU ($P<0.05$), which may confounded by comparing one case's peak to another's latency or recovery. To ensure serum N-Ag are comparable among cases, we selected 99 cases which serum samples were collected 3–7 days from symptom onset, a peak time period verified by our kinetics analysis. By matching the kinetics stage, our study provides more solid evidence supporting the correlation of serum N-Ag with disease severity.

This study has its own limitations and leaves points for further investigations. Although our preliminary data indicated the close correlation of serum N-Ag with disease severity, it needs further confirmation in focused clinical studies. As a retrospective study, we have limited ability to sample convenience specimens and capture data documented for clinical care. A prospective study with standardized capture of clinical data and outcomes would be more appropriate to evaluate utility in predicting disease severity. For asymptomatic cases with RT-PCR positive, we noticed different subgroups, including those with serum N-Ag positive (~31.6%), serum N-Ag and Abs negative (~42.1%), serum N-Ag negative and Abs positive (~26.3%). They likely indicate different stages of SARS-

CoV-2 infection. Further studies in asymptomatic populations will be required to confirm these findings and validate the use of serum N-Ag in disease temporalization.

In summary, our study validates serum N-Ag as a biomarker for SARS-CoV-2 acute infection with high sensitivity and specificity compared to viral RNA in the respiratory system. Moreover, considering the correlation between N-Ag level and disease severity, as well as the inverse relationship of N-Ag and Abs, serum N-Ag could be a potential biomarker for temporalizing disease or monitoring disease progress, for which further studies are warranted.

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Table 1. The kinetics of serum N-Ag by days from symptom onset.

From Symptom onset	Samples (n=)	Median (pg/mL)	IQR 25–75% (pg/mL)	TP (n=)	FN (n=)	Sensitivity
Day 0	26	1	1-167	11	15	42.3%
Day 1	22	18	1-211	15	7	68.2%
Day 2	22	116	30-2015	19	3	86.4%
Day 3	25	1038	59-3115	24	1	96.0%
Day 4	22	3520	1228-6741	21	1	95.5%
Day 5	17	1905	27-7091	17	0	100.0%
Day 6	8	5102	1875-10707	8	0	100.0%
Day 7	27	1731	680-9881	26	1	96.3%
Day 8-14	27	437	31-3416	23	4	85.2%
Day 15-21	7	144	1-13694	4	3	57.1%

n, case number; IQR, interquartile range; TP, True positive; FN, false negative.

Table 2. Sensitivity and specificity of Serum N-Ag during day 1–7 from symptom onset.

	NP swab-PCR positive (n=143)	NP swab-PCR negative (n=60)
Serum N-Ag positive	130	1
Serum N-Ag negative	13	59
	Sensitivity: 90.9% (95% CI, 85.1-94.6%)	Specificity: 98.3% (95% CI, 91.1–99.9%)

NP swab, nasopharyngeal swab; CI, confidence Interval.

Figure Legends

Figure 1A Kinetics of serum N-Ag concentrations by days after symptom onset. Each dot indicates one serum sample which was collected on the same day of NP swab collection for RT-PCR; each red line indicates the mean value in each time-period group. Cut-off was set at 2.97 pg/mL.

Figure 1B The Receiver operating characteristic (ROC) curves for serum N-Ag within the indicated weekly time frames. Area under the ROC Curve (AUC) are 0.961 (day 1–7), 0.925 (day 8–14), and 0.782 (day 15–21).

Figure 2 The correlation of serum N-Ag concentrations with disease severity (**A**) in cases with different highest level of care, including asymptomatic, symptomatic but discharged, hospitalized-to-non-ICU, and hospitalized-to-ICU; (**B**) in cases without medical interventions, with non-invasive oxygenation (NIO), or with mechanical ventilation (MV); (**C**) in cases without or with abnormal chest imaging; and (**D**) in cases with different length of hospital stay, including 0, 1-10, 11-20 and > 20 days. All serum samples were collected during day 3-7 from symptom onset. *, P< 0.05; **, P< 0.01; ***, P< 0.001; ****, P < 0.0001. The case number (n) is indicated under each group.

Figure 3A Kinetics of N-Ag concentrations and immunoglobulin M (IgM) and immunoglobulin G (IgG) responses for 20 hospitalized patients by days after symptom onset. For intensive-care-unit (ICU) cases, all cases with ≥7 time points and at least 1 time point >21 days were included. For non-ICU cases, all cases with ≥7 time points were included regardless of sample collection date.

Figure 3B-3C The inverse relationship between N-Ag and IgM (**B**) and IgG (**C**) in the 20 cases. To calculate the relative level in each case, Ag and Ab concentrations were normalized to the highest one found in each case.

Figure 1 Zhang et al.

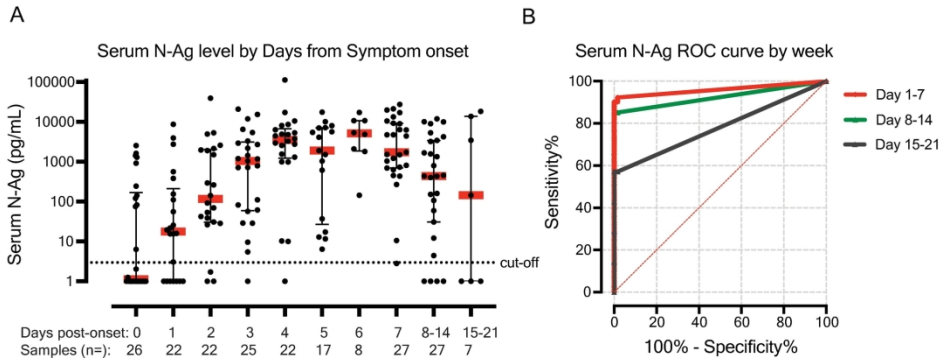

Figure 1 (A) Kinetics of serum N-Ag concentrations by days after symptom onset. Each dot indicates one serum sample which was collected on the same day of NP swab collection for RT-PCR; each red line indicates the mean value in each time-period group. Cut-off was set at 2.97 pg/mL. Any Ag concentration under 1 pg/mL, lower than limit of blank (1.08 pg/mL), was transferred to 1 pg/mL for data representation on the log-scale. (B) The Receiver operating characteristic (ROC) curves for serum N-Ag within the indicated weekly time frames. Area under the ROC Curve (AUC) are 0.961 (day 1–7), 0.925 (day 8–14), and 0.782 (day 15–21).

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Figure 2 Zhang et al.

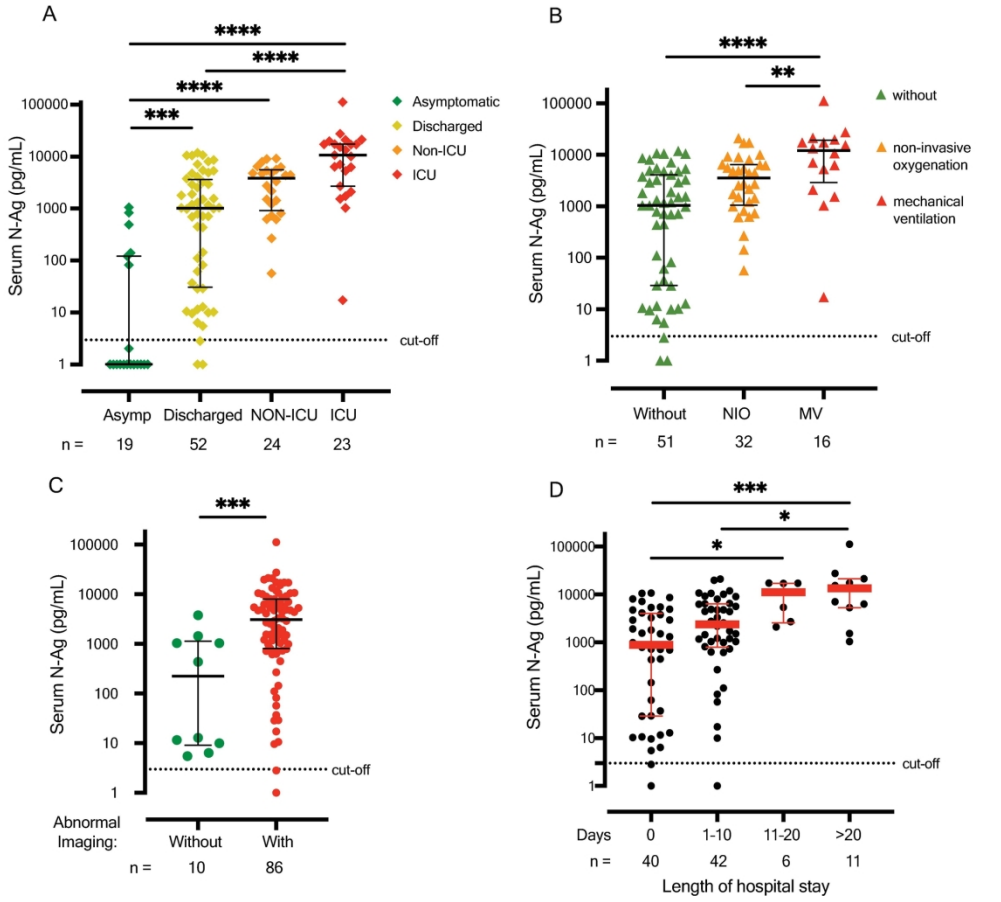

Figure 2 The correlation of serum N-Ag concentrations with disease severity (A) in cases with different highest level of care, including asymptomatic, symptomatic but discharged, hospitalized-to-non-ICU, and hospitalized-to-ICU; (B) in cases without medical interventions, with non-invasive oxygenation (NIO), or with mechanical ventilation (MV); (C) in cases without or with abnormal chest imaging; and (D) in cases with different length of hospital stay, including 0, 1-10, 11-20 and > 20 days. All serum samples were collected during day 3-7 from symptom onset. *, P< 0.05; **, P< 0.01; ***, P< 0.001; ****, P < 0.0001. The case number (n) is indicated under each group. Any Ag concentration under 1 pg/mL was transferred to 1 pg/mL for the data representation on the log-scale.

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Figure 3 Zhang et al.

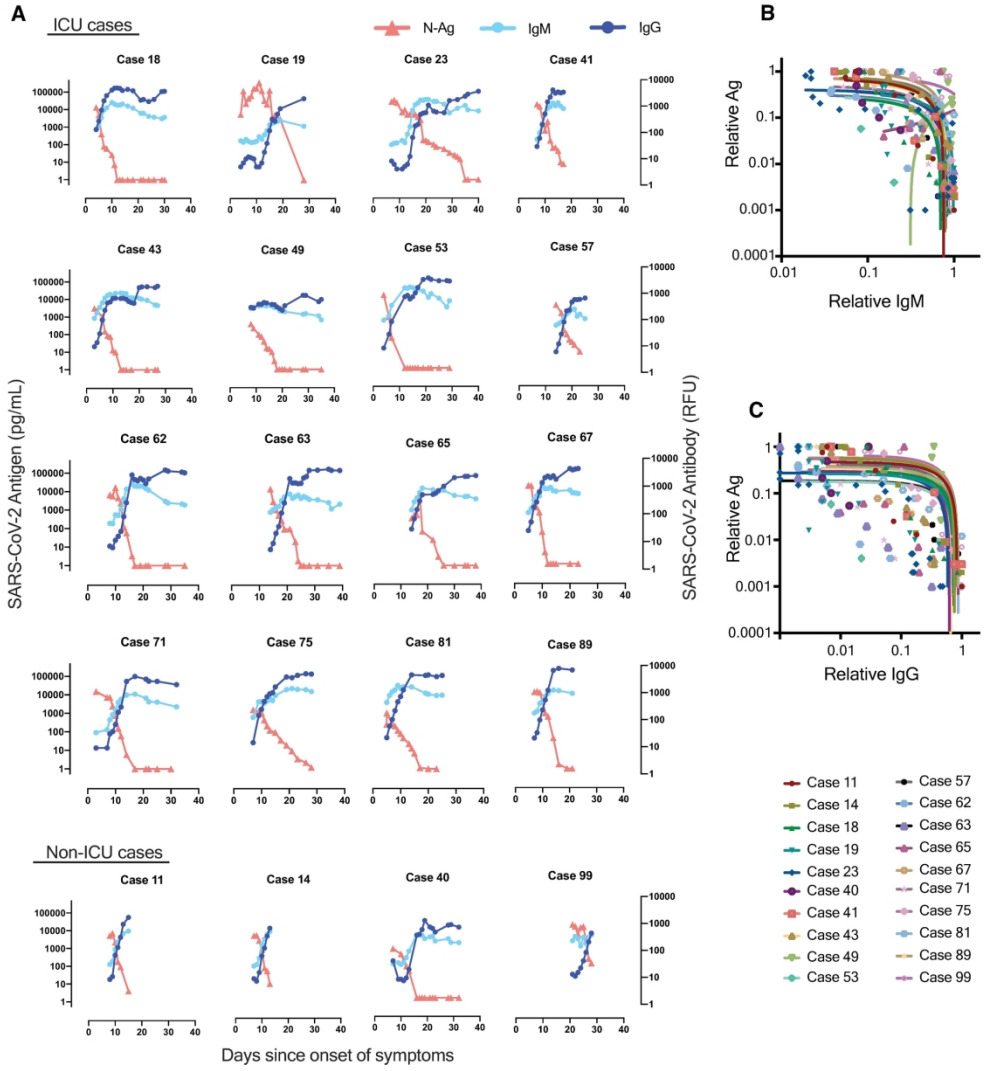

Figure 3 (A) Kinetics of N-Ag concentrations and immunoglobulin M (IgM) and immunoglobulin G (IgG) responses for 20 hospitalized patients by days after symptom onset. For intensive care unit (ICU) cases (N = 16), all cases with ≥7 time points and at least 1 time point >21 days were included. For non-ICU cases (N = 4), all cases with ≥7 time points were included regardless of the time of sample collection since symptom onset. Any Ag value under 1 pg/mL was transferred to 1 pg/mL for the data representation on the log-scale. (B and C) The inverse relationship between N-Ag and IgM (B) and IgG (C) in the 20 cases. To calculate the relative level in each case, Ag and Ab concentrations were normalized to the highest one found in each case.

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Figure S1

Zhang et al.

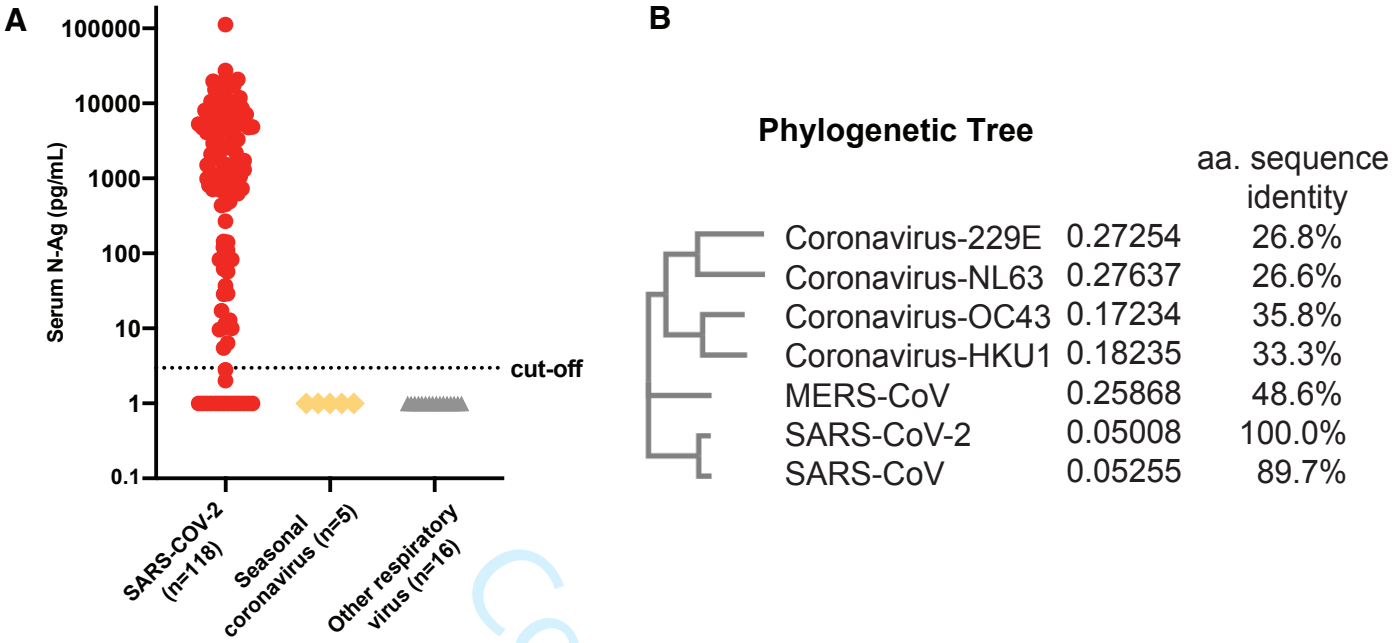
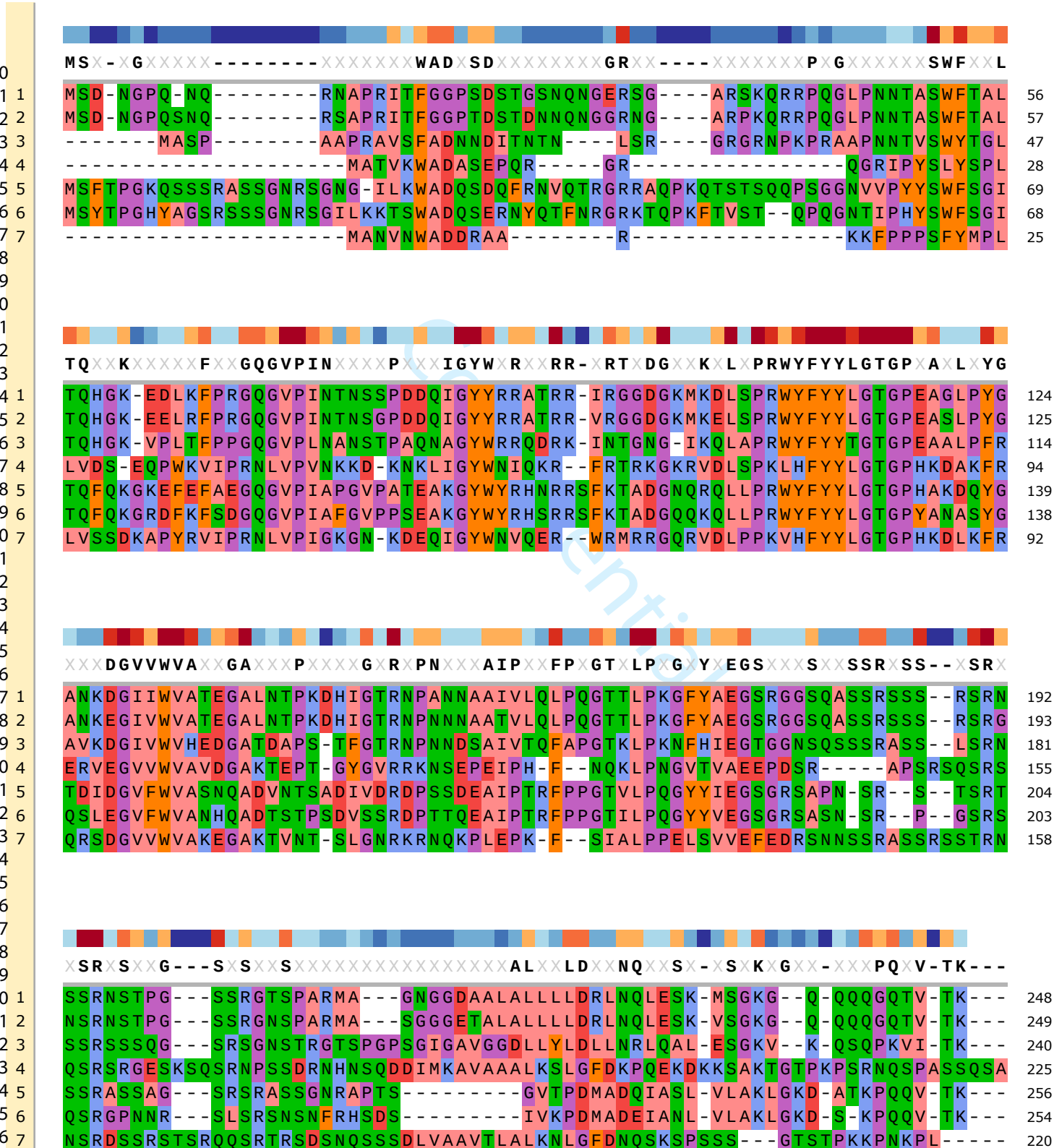


Figure S1 (**A**) Serum N-Ag concentrations observed in cases infected with SARS-CoV-2 (N=118), seasonal coronavirus (N=5), and other respiratory virus (N=16). SARS-CoV-2 cases include 99 symptomatic cases on day 3-7 from symptom onset and 19 assymptomatic cases. Seasonal coronavirus includes 229E, OC43, and HKU1; other rspiratory virus includes human rhinovirus /enterovirus, metapneumovirus, respiratory syncytial virus, parainfluenza type 1 virus, and adenovirus. Any Ag concentration under 1 pg/mL, lower than limit of blank (1.08 pg/mL), was transferred to 1pg/ml for the data representation on the log-scale. **(B)** The amino acid (aa.) sequence similarity of N proteins within the coronavirus family.



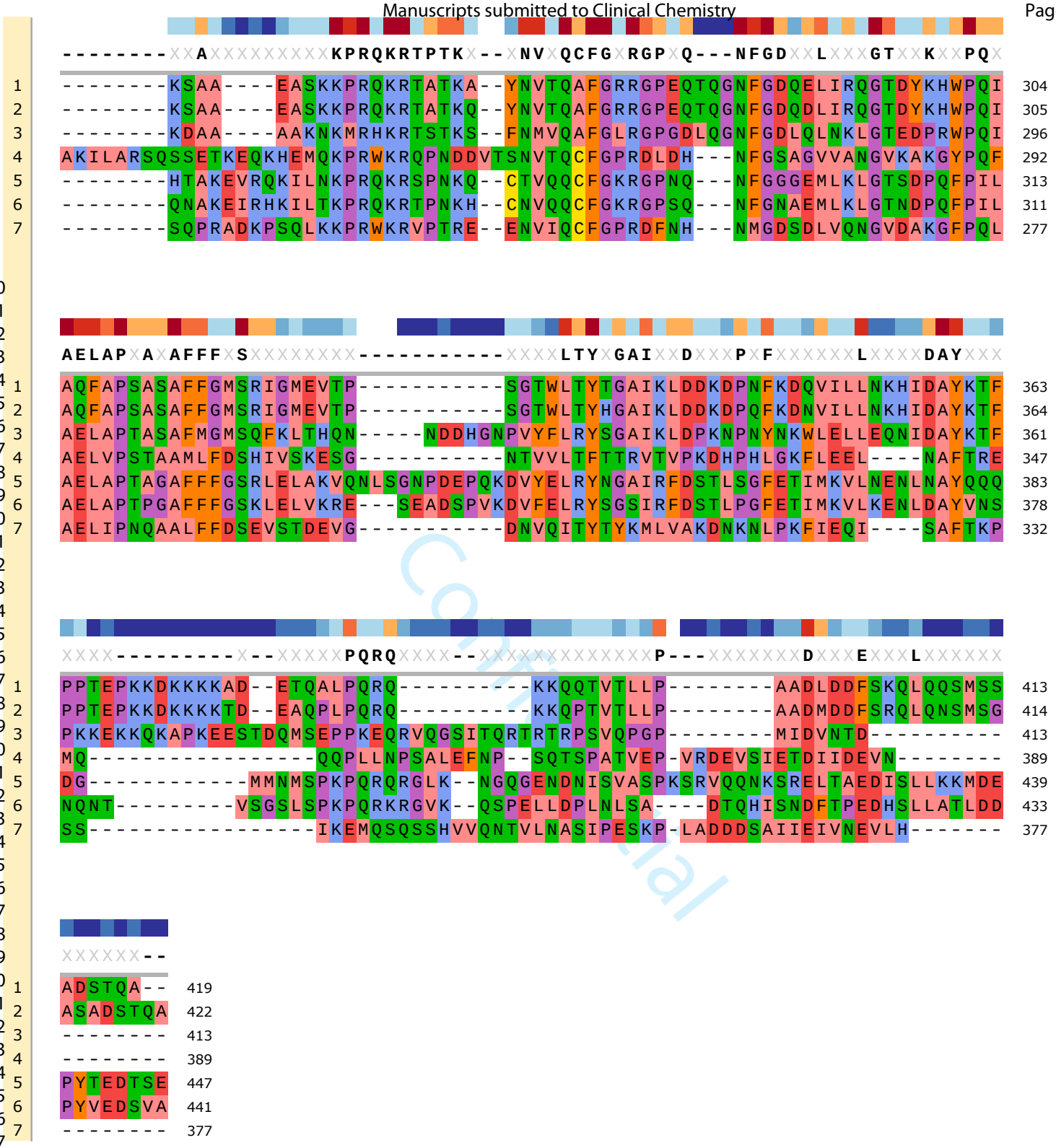


Figure S2

Zhang et al.

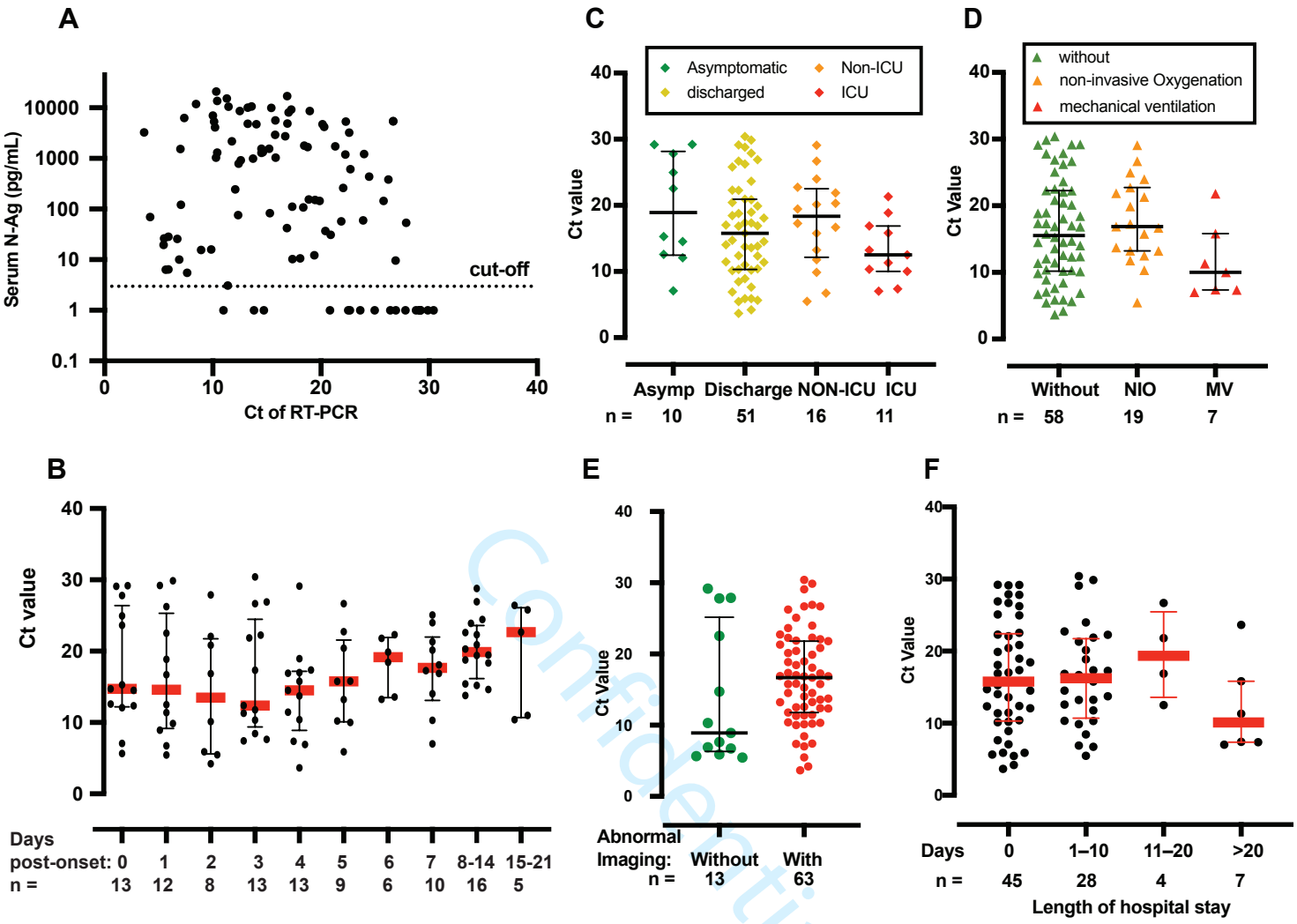


Figure S2 (**A**) No significant correlation between N-Ag concentrations in serum and Ct values of RT-PCR in NP swabs. Each dot indicates one case, for which serum and NP swab samples were collected within 24 hrs. Cut-off was set at 2.97 pg/mL. Any Ag concentration under 1 pg/mL, lower than limit of blank (1.08 pg/mL), was transferred to 1 pg/mL for data representation on the log-scale. (**B**) Ct value by days after symptom onset. Each dot indicates Ct value of RT-PCR which evaluated viral RNA in a NP swab sample and were grouped by swab collection days from symptom onset, and each red line indicates the mean value in each time-period group. (**C**-**F**) No significant correlation of Ct Values of RT-PCR with disease severity (**C**) in cases with different highest level of outcomes, including asymptomatic, symptomatic but discharged, hospitalized-to-non-ICU, and hospitalized-to-ICU; (**D**) in cases without medical interventions, with non-invasive oxygenation (NIO), or with mechanical ventilation (MV); (**E**) in cases without or with chest abnormal findings; and (**F**) in cases with different length of hospital stay, including 0, 1-10, 11-20, and > 20 days. All swab samples were collected during day 0-7 from symptom onset. The case number (n) is indicated under each group.

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