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Ph. D. Dissertation

Clinical Usefulness of SARS-CoV-2 Detection and Diagnosis in Patients with COVID-19

Graduate School of Chosun University

Department of Biomedical Sciences

Merlin Jayalal Lawrence Panchali

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코로나19 환자에서 SARS-CoV2 검출 및
진단의 임상적 유용성

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초 록 (ABSTRACT)

코로나19 환자에서 SARS-CoV-2 검출 및 진단의 임상적 유용성

멀린 자야랄 로렌스 판찰리
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배경: 코로나바이러스감염증 (COVID-19)은 코로나 19 바이러스 (SARS-CoV-2)로 인해 발병하며, 무증상 에서부터 중증까지 발현되는 호흡기 질환이다. COVID-19 진단에는 PCR검사를 이용한 분자진단과 항원항체검사를 이용한 면역학적진단법이 이용된다. PCR진단에 CDC 또는 WHO에서 권장하는 프라이머를 이용한 실시간 역전사 중합효소 연쇄 반응(RT-qPCR)이 이용되나 임상적 유용성을 비교한 연구는 거의 없다. 바이러스 RNA 혈증 및 항원 혈증의 임상적 유용성 또한 아직 입증되지 않았다. 이 연구는 SARS-CoV-2 RNA혈증, 항원 혈증및 변이에 따른 진단 정확도의 평가를 통해 SARS-CoV-2 진단에 사용되는 항원검사의 임상적 유용성에 대한 연구를 수행하고자 하였다.

방법: 실시간 역전사 중합효소 연쇄 반응(RT-qPCR)의 정확도 평가를 위해 WHO 및 CDC에서 권장하는 프라이머 및 다양한 상용 프라이머와 함께 연구자가 디자인한 뉴클레오캡시드 단백질을 표적으로 하는 프라이머 세트(iNP)를 제작하여 비교하였다. COVID-19 확진은 바이러스가 배양되거나 항체가 4배 상승된 경우로 정의하였다. 환자의 혈청 샘플의 바이러스 RNA copies와 항원농도를 확인하여 COVID-19 환자의 임상적 유용성을 평가하였다. 상부 및 하부 호흡기 검체를 이용하여 야생형, 델타 및 오미크론 변이체에 따른 COVID-19 환자의 SARS-CoV-2에 대한 iNP RT-qPCR 의 진단 정확도를 평가하였다.

결과: 사이클 역치(Ct)의 컷오프 값을 35로 설정했을 때 WHO RdRp 프라이머와 CDC의 N1, N2, N3 프라이머를 사용한 RT-qPCR결과 객담에서 민감도 42.1~63.2%, 특이도 90.5~100%, 비인두 검체에서 민감도 65.2~69.6%, 특이도 65.2~69.6%를 보였다. 객담을 이용할 경우 민감도가 가장 높았고 비인두, 타액, 구인두 검체의 순서를 보였다. (P = 0.0193). 정확도 평가 연구 결과 iNP RT-qPCR이 WHO(P < 0.0001) 또는 CDC(N1: P = 0.0012, N2: P = 0.0013, N3: P = 0.0012) 프라이머를 사용한 RT-qPCR보다 더 나은 민감도와 특이도를 확인하였다. RNA혈증의 유무는 위중하거나 치명적인 환자군에서 가장 높았고(66.7%), 중증(12.5%) 및 경증 내지 중등도(1.7%)순 이였다. 입원 및

1주차 검체에서 바이러스성 RNA혈증이 검출되었으나, 증상 발현 후 2주차에 채취한 검체에서는 RNA혈증이 검출되지 않았다. 다중회귀분석은 RNA혈증이 질병의 중증도에 대한 독립적인 예측인자임을 보여주었고(P = 0.021), Kaplan-Meier 생존 곡선 검사상 추적 검체에서 항원 혈증 농도가 증가할 때 더 높은 사망률을 보였다(P = 0.005). 단변량 분석에서는 연령, PSI, 상승된 항원 혈증 및 RNA혈증이 사망의 예측 위험인자로 나타났고 다변량 로지스틱 회귀 분석에서는 나이 및 RNA혈증이 사망의 위험인자로 나타났다. 변이에 따른 진단 정확도 평가에서 iNP 유전자 RT-qPCR 검사 결과, 오미크론 변이 환자의 타액 샘플을 이용한 민감도가 델타 변이체(AUC-0.875) 및 야생형(AUC-0.878) 타액 샘플과 비교하여 더 높은 민감도(AUC-1.000)를 보였다. 그러나 SARS-CoV-2 오미크론 변이체에 감염된 환자의 백신 접종 또는 미접종 환자의 타액 샘플에서 바이러스 양에 유의한 차이는 없었다.

결론: SARS-CoV-2 에 대한 RT-qPCR 분석에서 가장 높은 민감도를 보이는 것은 객담 검체였고 비인두, 타액, 구인두 검체의 순서를 보였다. 또한 SARS-CoV-2의 검출에서 연구자가 디자인한 RT-qPCR 의 정확도가 훨씬 높아 WHO 및 CDC 프라이머 세트에 대해 개선이 필요한 것으로 생각된다. SARS-CoV-2 감염의 RNA혈증은 COVID-19 환자의 임상 중증도에 대한 위험 예측 인자임을 확인했다. 항원 혈증 농도의 상승과 혈액 내 RNA혈증 바이러스 부하가

사망률과 상관관계를 입증하였다. 또한 오미크론 변이의 타액 샘플이 야생형 및 델타 변이 타액 샘플보다 더 나은 민감도를 가지고 있음을 확인하였다.

ABSTRACT

Clinical usefulness of SARS-CoV2 antigen detection and diagnosis in Patients with COVID-19

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Background: Coronavirus disease 2019 (COVID-19), a mild to severe respiratory illness caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The diagnostic accuracy of real-time polymerase chain reaction (RT-qPCR) primers in clinical practice remain unproven which includes CDC- and WHO-recommended primers. However, the clinical relevance of RNAemia and antigenemia has not been well documented in the literature. In this study I aimed to examine the clinical relevance of SARS-CoV-2 RNAemia and nucleocapsid protein antigenemia in association with COVID-19 severity. In addition kinetics of viral load in various respiratory samples were tested for diagnostic accuracy according to variants of SARS-CoV-2 in COVID-19 patients.

Methods: The accuracy of reverse transcription-polymerase chain reaction (RT-qPCR) were analyzed using an in-house–designed primer set (iNP) targeting the nucleocapsid protein along with WHO and CDC recommended primers and various commercial primers. The accuracy was also assessed by virus culture or seroconversion. Furthermore, we explored a cohort study of COVID-19 patients and their prospects with evidence of RNAemia using RT-qPCR in serum. In addition I explored the kinetic responses of antigenemia, RNAemia, and viral loads in various respiratory tract specimens in COVID-19 Patients along with various clinical characteristics. Moreover I analyzed the correlation of various risk factors with COVID-19 mortality. I also studied the viral load kinetics and diagnostic accuracy of COVID-19 patients with respective of SARS-CoV-2 variants, which includes wild-type, delta and omicron variants using RT-qPCR assay using upper and lower respiratory tract specimens.

Results: When a cutoff value of the cycle threshold (C_t) was set to 35, RT-qPCRs using WHO RdRp primer and CDC N1, N2, and N3 primers showed sensitivity 42.1–63.2% and specificity 90.5–100% in sputum and sensitivity 65.2–69.6% and specificity 65.2–69.6% in nasopharyngeal samples. Sputum testing had the highest sensitivity, followed by nasopharyngeal testing ($P = 0.0193$). Our results suggest that iNP RT-qPCR has better sensitivity and specificity than RT-qPCR with WHO ($P <$

0.0001) or CDC (N1: P = 0.0012, N2: P = 0.0013, N3: P = 0.0012) primers. The presence of RNAemia in critical or fatal cases was the highest (66.7%), followed by severe (12.5%) and mild to moderate (1.7%) in admission samples. Viral RNAemia was detected on admission and 1st week samples, however, RNAemia was not detected on the samples collected on the second week post-symptom onset. Multiple regression analysis showed that the RNAemia was an independent predictor for the disease severity (P = 0.021), and the Kaplan-Meier survival curve estimated an increased mortality rate in COVID-19 RNAemia cases (P < 0.001). In addition, the presence of antigenemia in asymptomatic patients on admission and 1st week were 27% and 22%, however none of the samples collected on 2nd week possess any antigenemia. Kaplan–Meier survival curves predicted a higher mortality rate when there is an elevated concentration of antigenemia in follow-up samples (P = 0.005). Univariate analysis designates that age, PSI, elevated antigenemia and RNAemia were predictive rick factors of mortality and with multivariate logistic regression analysis, age and RNAemia were risk factors of mortality. In SARS-CoV-2 variant diagnostic detection the sensitivity/specificity of *NP* gene RT-qPCR results of saliva sample of omicron variant has higher sensitivity (AUC- 1.000) compared with delta (AUC- 0.875) and wild-type type (AUC- 0.878) saliva samples. Our results reports that there is no significant difference in the viral load in saliva samples of vaccinated or non-vaccinated patients infected with SARS-CoV-2 omicron variants.

Conclusions: In conclusion, I demonstrated that sputum RT-qPCR analysis has the highest sensitivity, followed by nasopharyngeal, saliva, and oropharyngeal samples. We also suggests that considerable improvement is needed for RT-qPCR WHO and CDC primer sets in detection of SARS-CoV-2. In addition, RNAemia of SARS-CoV-2 infection is a predictive risk factor for clinical severity in COVID-19 patients. Furthermore we demonstrated the potential relation and correlation between the antigenemia concentration and RNAemia viral load in blood in the mortality outcome. Our study suggests that the saliva sample of omicron variant have better sensitivity than wild-type type and delta variant saliva samples. However there is no significant difference in viral load of saliva samples in vaccinated or non-vaccinated patients of delta and omicron variant infected patients with COVID-19.

I. INTRODUCTION

1.1. Background of SARS-CoV-2

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the seventh identified coronaviruses in the last 20 years among the ninth documented coronavirus that cause humans infections [1]. SARS-CoV-2 which cause COVID-19 with unexplained pneumonia were observed first in Wuhan city, Hubei Province, in late 2019 [2]. Henceforth the COVID-19 spread rapidly to all 34 provinces of China with daily thousands of cases in late January 2020 [3]. WHO declared a public health emergency of international concern on January 30, 2020 [4]. On 11th March 2020 WHO officially characterized the COVID-19 outbreak as a global pandemic [5]. Among all RNA viruses, SARS-CoV-2 is the largest RNA genomes virus having about 30 kilo base nucleotides encoding for about 29 proteins mainly with four structural proteins namely, spike protein (S), nucleocapsid protein (N), envelope protein (E), and membrane protein (M) [6]. Full-length aligned genome sequence of SARS-CoV-2 with other available beta coronaviruses genomes indicated that SARS-like coronavirus strain from the bat (BatCov RaTG13) had 96% identity relationship with SARS-CoV-2 suggesting that bat may be the origin SARS-CoV-2 which evolved naturally from BatCov RaTG13 strain [7]. In an another study, coronavirus isolated from Malaysian pangolin has the high genetic similarity within *E-gene, M-gene, NP-gene, and Spike-gene* with 100, 98.6, 97.8, and 90.7%, suggesting

pangolins might be an intermediate host of SARS-CoV-2 viral transmission to humans [8]. SARS-CoV-2 viral transmission occurs mainly from person-to-person form places which includes hospital, community gathering, family and other gathering of peoples [9, 10].

1.2. Clinical manifestations and characteristics of COVID-19

The COVID-19 clinical manifestations include fever, cough, and fatigue; whereas, few patients experience nasal congestion, runny nose, dyspnea, and diarrhea with bilateral pneumonia in majority of the patients, whereas SARS-CoV-2 comorbidities more likely affected in older patients [11]. Similarly, respiratory failure and longer disease course period were observed with patients older than 60 years, and the severity is milder in patients under 60years of age [12]. The case-fatality rate (CFR) was 8.0% in age 70−79 years, whereas the highest CFR of 14.8% was observed in patients above 80 years old, meantime patients having comorbidities such as diabetes, cardiovascular disease, hypertension, chronic respiratory disease, and cancer possess higher CFR [13]. Lungs is the primary target of infection which followed by cardiovascular, kidney, liver and immune systems infection and most significantly of COVID-19 is the life threatening acute respiratory distress syndrome (ARDS) [14, 15]. The clinical manifestation in CT were bilateral, peripheral, and ground-glass opacity were most common and the findings with nodules, cystic changes, pleural

effusion, and lymphadenopathy were less common [16]. Bilateral lungs with diffuse lesions was seen in the most critical patients, whereas the patients CT observed as a white lungs [17]. COVID-19 disease severity was classified to asymptomatic, mild, severe, and critical or fatal as presented in Fig 1 in accordance with age [18]. The viral transmission from asymptomatic or pre-symptomatic patient causes difficulty in control measures, which include hygiene enhancement and contact tracing [19].

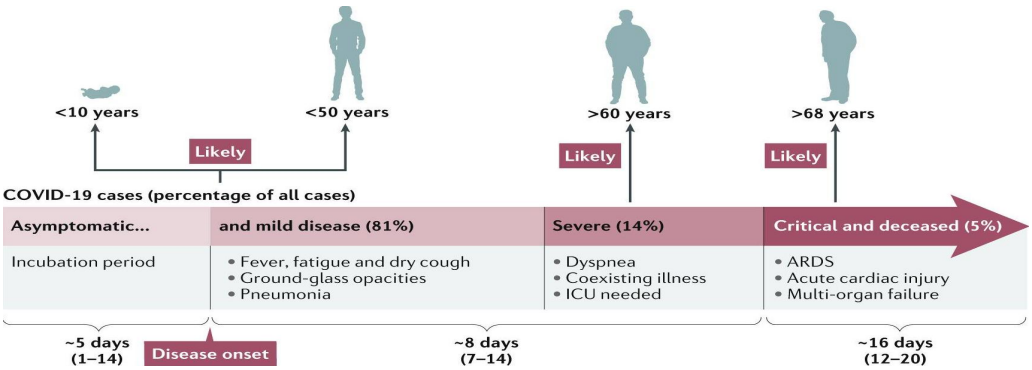


Figure 1: Clinical features and classification of COVID-19 patients (*18*)

1.3. Viral shedding and diagnosis of SARS-CoV-2 in COVID-19 patients

The Real-time reverse transcription–polymerase chain reaction (RT–qPCR) was the primary step in rapid and accurate detection of SARS-CoV-2 in managing COVID-19 infection with constant improvement in test sensitivity [20]. For diagnosis of COVID-19 the crucial factor was selecting the appropriate specimen

(Fig 2) to detect the infection, in general the nasopharyngeal swab of upper respiratory tract were usually tested, however in some instances due to false negative results lower respiratory tract specimens such as sputum, bronchoalveolar lavage (BAL) were considered as alternative samples [21]. Hence it is important to collect various samples from suspected SARS-CoV-2 infected individuals which can reduce the false negative results and advocated different specimen types for SARS-CoV-2 infection for testing and to monitor the disease progression and prognosis [22]. Furthermore self-collected saliva specimens from patients suggested that it's a non-invasive specimen for monitoring, infection control and for the diagnosis of SARS-CoV-2 [23]. Viral RNA were present in the swab samples of recovered patients for about 50 days and also present in fecal and anal swabs of patient's after respiratory specimens were negative using RT-qPCR [24]. Viral shedding was observed for a minimum of 7 days after symptom onset whereas the viral infectivity was noted within 24 hrs of contact with the virus, however the detection rate starts to decline parallel to the increase in serum neutralizing antibodies [25].

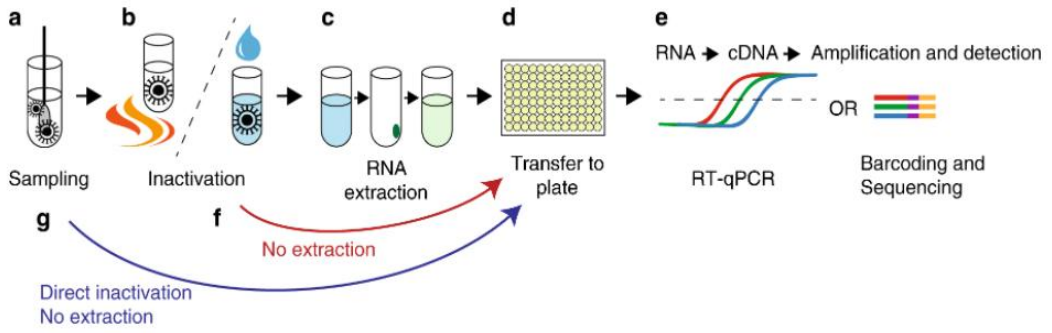


Figure 2: A systematic representation of RT-qPCR testing (*Nat Commun.* 2020 Sep 23;11 (1):4812)

COVID-19, RT-qPCR diagnosis was developed mainly based on the following target genes namely E gene, N gene, and S gene and the RNA-dependent RNA polymerase (RdRp) gene of the SARS-CoV-2 genome [26,27,28]. The detection of serum IgM/IgG antibody against the SARS-CoV-2 surface spike protein, internal nucleoprotein (NP) and receptor-binding domain (RBD) can compensate the false positive or negative diagnosis with RT-qPCR. IgM antibodies tends to rise within a few days of infection and IgG antibodies tends to appear in later stage of infection and continues to increase the titer and remains in circulation for prolonged period of time [29]. Enzyme-linked immunosorbent assay (ELISA), Immunofluorescence assay (IFA) and convalescent-phase sera by colloidal gold immunochromatography were widely used for the antibody detection in acute and convalescent-phase sera and were used to determine the infection of SARS-CoV-2 occurred recently or formerly [30]. Several nonspecific detection and examination were also carried out

in different circumstances, where in severe cases an abnormal level of lactate dehydrogenase, C-reactive protein, alanine aminotransferase, ferritin, and D-dimer together with and increased levels of IL-2R, IL-6, IL-10, and TNF-α were also observed [31]. Moreover several rapid antigen test kits were developed to identify the viral antigens of SARS-CoV-2 in different clinical specimens to identify the early infections [32]. Active SARS-CoV-2 viral culture were used in the isolation of live virus and cytopathic assays, whereas most importantly used in the identification of live virus in immunocompromised patients where the infectious virus were found for prolonged period of time [33].

1.4. SARS-CoV-2 demographic factors, laboratory indicators, social and lifestyle risk factors in COVID-19 patients

Numerous factors had posited the risk of COVID-19 which includes biological risk factors, medical risk factors, social risk factors such as low socioeconomic status, crowded housing, and necessary use of public transportations [34]. During the hard hit COVID-19 pandemic time in USA reports suggested that there were longstanding burdens by social determination of health with racial and ethnic origin of patients [35, 36, 37]. Higher mortality rates were observed in African American, Hispanic, and Native American with SARS-CoV-2 infections [38, 39]. Multiple studies from China, Korea, Italy, The United Kingdom, and USA presented evidence of higher

risk with age-associated vulnerability to SARS-CoV2 infection with increased mortality and severity in older patients [40]. In addition females have the lesser prevalence of SARS-CoV-2 infection, whereas males having higher prevalence of infection with higher CFR rate in relatively younger age patients with COVID-19 [41]. Furthermore lifestyle risk factors such as, smoking and obesity were well known in correlation of severe illness and higher case fatality rate [42]. In a study conducted using mendelian randomization (MR) to investigate the causality of COVID-19 patients according to body mass index, physical activity and smoking suggested that an increased physical activity decrease the severity whereas an increased body mass index and smoking pawed way for severe illness [43, 44, 45].

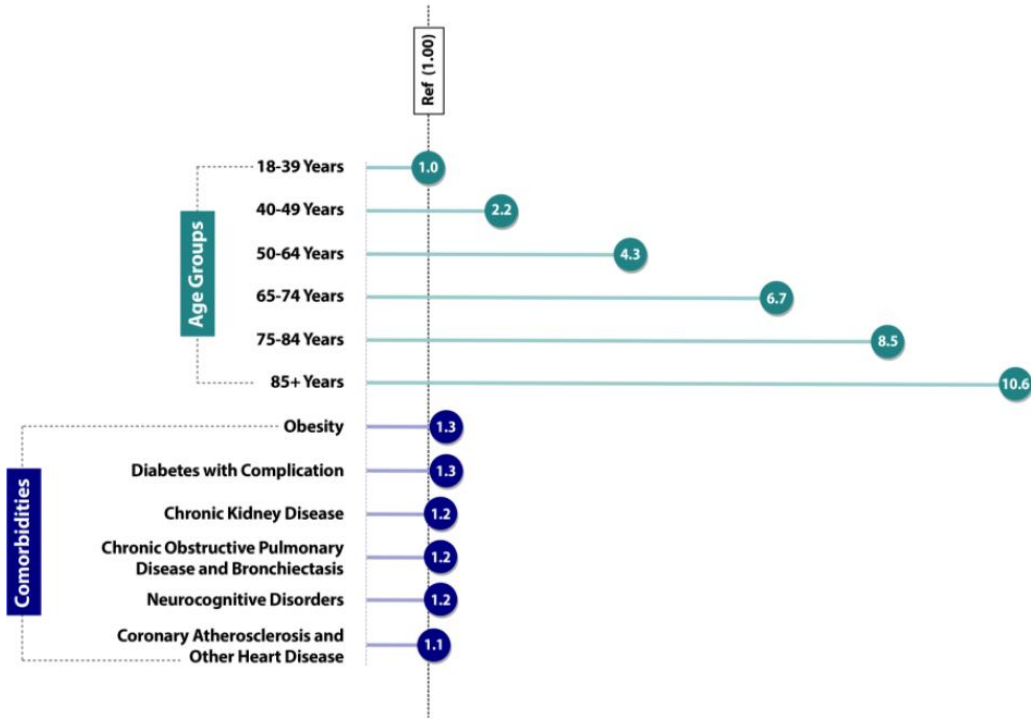


Figure 3: COVID-19 mortality ratio according to age and comorbidity (https://www.cdc.gov/coronavirus/2019-ncov/hcp/clinical-care/underlyingconditions)

Higher risk of severity and mortality were observed in patients with underlying medical conditions such as. cancer, chronic kidney disease, chronic lung diseases limited to interstitial lung disease, pulmonary embolism, pulmonary hypertension, bronchiectasis and COPD (chronic obstructive pulmonary disease), chronic liver diseases limited to: cirrhosis, non-alcoholic fatty liver disease, alcoholic liver disease, autoimmune hepatitis, cystic fibrosis, diabetes mellitus with type 1 and type 2, heart conditions (such as heart failure, coronary artery disease, or cardiomyopathies) infected with SARS-CoV-2 [46]. Various studies had effectively predicted the

disease severity and poor prognosis in patients using laboratory indicators with the patients infected with COVID-19. A sensitive biomarker for inflammation, C reactive protein (CRP) has been identified as an independent risk factor for the progression of COVID-19 [47]. In addition Neutrophil-to-lymphocyte ratio (NLR) has been predicted as a severity factor with high accuracy in SARS-CoV-2 infected patients [48, 49]. Several studies had predicted that there is significant increase in D-dimer due to inflammatory response leads to higher severity and mortality in COVID-19 patients [50].

II. OBJECTIVE AND SCOPE

2.1. Objective and scope of the present study

The prime objective of the current study is to establish a concrete evidence on the prognostic factors which portrays the prediction of disease severity in diagnosing SARS-CoV-2 infection in COVID-19 patients. In order to demonstrate the prognostic factors, first we evaluated the diagnostic accuracy of RT-qPCR with various primer sets along with an in-house designed (iNP) primer sets. Furthermore we assayed the specificity and sensitivity of the various primers including WHO and CDC recommended primer sets which were used in worldwide for diagnosis. In-order to study the accuracy, the samples collected from the patients were subjected to RT-qPCR in accordance with various body specimens in time dependent manner.

Next we study the effect of prognostic factors of SARS-CoV-2 infection and disease severity. Here we evaluated the clinical implication of RNAemia and antigenemia individually and antigenemia in association with RNAemia. We elaborated and demonstrated the SARS-CoV-2 predictive risk factors in disease severity and mortality of COVID-19 patients.

Moreover we attempted to study the viral kinetics on COVID-19 patients infected with different SARS-CoV-2 variants. Here we elaborated the diagnostic sensitivity of wild-type, delta and omicron variants in accordance with saliva samples and viral

kinetics variation with vaccinated and non-vaccinated patients. The present research work will narrow the gap in understanding the prognostic factors in disease severity and mortality outcome of COVID-19 patients. Moreover our results will also provide a valuable insight on viral kinetics with different variants in accordance with different samples and vaccination status.

2.2. Diagnostic accuracy of SARS-CoV2 real-time polymerase chain reaction

Real-time reverse-transcriptase polymerase chain reaction (RT-qPCR) tests, was considered as a reference standard to detect the SARS-CoV-2 viral RNA in clinical samples [51]. Several RT-qPCR based techniques have been approved for early diagnostic of SARS-CoV-2 infection. Nevertheless, swab samples including nasopharynx and oropharynx samples PCR resulted in varied positivity rates in COVID-19 patients [52, 53]. However the most widely used Nucleic Acid Amplification Test (NAAT) might leads to false negative and false positive results in the diagnosis which can leads to grave consequences in virus transmission [54, 55, 56].

In a previous study the efficiency of IgM using enzyme-linked immunosorbent assay (ELISA) for detection is better than real-time PCR (RT-qPCR) after 5 days of

symptom onset [57]. A fourfold increase in a SARS-CoV-2 antibody titer or a culture positive is believed to be required for further confirmation as using gold standard [58, 59]. Only a few studies have elaborated the sensitivity and specificity of which samples may have the highest potential for the accurate detection of SARS-CoV-2 in COVID-19 patients. In the current study we validated the sensitivity and specificity of RT-qPCR using various primer sets including WHO and CDC primers and explored the most sensitive specimen for clinical diagnosis of SARS-CoV-2 in COVID-19 patients.

2.3. SARS-CoV-2 RNAemia as a prognostic factor in disease severity on COVID 19 patients

Prognostic importance of viremia (viral RNA in blood means RNAemia) on disease progression had gained attention in the later stage of COVID-19 pandemic, where only a few studies have examined the viral load in peripheral blood and analyzed its importance [60, 61, 62, 63]. A previous study reported that the respiratory specimen viral loads of both symptomatic and asymptomatic patients had no differences, which indicates that the disease severity was not objectively depend on the respiratory specimen's viral load [64]. SARS-CoV-2 viral RNA was detected in 15% of the peripheral blood samples of COVID-19 patients in a previous reported study [65]. RNAemia kinetics of SARS-CoV-2 viral RNA in blood have been reported before;

however, the underlying prognostic efficacy is not fully understood weather RNAemia play a vital role in disease severity and mortality using follow-up studies [66, 67, 68]. In the current study, we validated the SARS-CoV-2 RNAemia kinetics and we quantitatively assessed the dynamics of blood RNAemia in clinically confirmed COVID-19 patients. In addition we utilized logistic regression analysis to identify weather RNAemia was a prognostic factor in prediction of disease severity and mortality.

2.4. SARS-CoV2 Antigenemia as a prognostic markers in COVID 19 patients

Identification of SARS-CoV-2 nucleocapsid antigen (antigenemia) had been reported before, but the prognostic implication of antigenemia and its role in disease severity is not understood [69]. Studies have found that a prolonged antigenemia in blood had masked the humoral immune response in a patient infected with COVID-19 [70]. SARS-CoV-2 antigenemia were found in higher percentage with the COVID-19 infected patients whereas higher proportion of SARSCoV□ 2 antigenemia found in ICU admission patients were linked with higher mortality rate [71, 72]. Another reported study suggested that *NP* protein antigenemia can be used as a clinical marker for screening epidemiological relations of asymptomatic infection of SARS-CoV-2 [73]. SARS-CoV-2 viremia and antigenemia only have

been observed and documented in disproportionally lower rates despite gaining more clinical significance and the prognostic parameters remains poorly understood [74, 75, 76, 77]. In the present study we analyzed the SARS-CoV-2 *NP* protein antigenemia and its role in disease severity and mortality outcome. Moreover we further analyzed the kinetics between the RNAemia and antigenemia in disease severity and mortality.

2.5. SARS-CoV-2 viral kinetics in accordance with different variants

Several SARS-CoV-2 variants have been emerged since the COVID-19 pandemic, which includes, alpha, beta, gamma, delta, eta, lota, kappa, lambda and omicron. Alpha was the first variant found in Kent, UK, Beta variant called B.1.351 was first identified Republic of South Africa and Delta variant called B.1.617.2 was first reported in India [78, 79, 80]. Recent variant of SARS-CoV-2, B.1.1.529 named as Omicron from South Africa was first reported on November 2021 with multiple mutations in spike protein with increased transmissibility [81]. Furthermore, different specimens testing gave negligible false-positive and false-negative results during the diagnosis of SARS-CoV-2 in COVID-19 patients [82, 83]. Saliva sample was used as an alternative sample for monitoring the viral shedding and viral load of SARS-CoV-2 in COVID-19 patients [84]. Here we explored the viral load kinetics in three different variants namely Wild type, Delta and Omicron variants. Moreover

we assayed the viral kinetics of different samples which includes upper (oropharyngeal, nasopharyngeal and saliva) and lower (sputum) respiratory tract specimens in different variants. In addition we studied the difference in viral load in COVID-19 patients with and without vaccination with respective to variants.

III. MATERIALS AND METHODS

3.1. Participants and data source

The clinical samples were obtained from patients clinically confirmed with COVID-19 at the Chosun University Hospital, South Korea. The clinical samples were further confirmed using an in-house RT-qPCR targeting *NP* protein. Furthermore for gene *E* (encoding envelope protein) and *RdRp* (encoding RNA-dependent RNA polymerase), Kogene Kit (Kogene Biotech Co., Ltd., Seoul, South Korea) and SD Kit (SD Biotechnologies Co., Ltd., Seoul, South Korea) were utilized. In addition for further confirmation other diagnostic methods such as cell culture, ELISA, and immunofluorescence assays (IFA) were performed as mentioned below. Moreover, to study the sensitivity and specificity samples were collected for healthy subjects, where the samples were collected before the pandemic or the subjects with no history of SARS-CoV-2 infection or no history of antibody detection were utilized.

3.2. SARS-CoV-2 specimen sampling from COVID-19 patients

Nasopharynx swabs, oropharynx swabs, sputum, saliva, urine, stool, serum/plasma, and whole blood were obtained from the patients for further clinical identification. Sputum, saliva, stool, and urine samples were self-collected by the patients. Upper respiratory track samples such as nasopharynx and oropharynx samples we collected

by a physician and directly transferred to UTM™ kits containing 1 mL of a viral transport medium (Noble Bio, Korea) and stored as per the manufactures instructions.

3.3. SARS-CoV-2 viral RNA extraction

Self-collected sputum samples from the patients were diluted in phosphate-buffered saline (PBS), mixed, and centrifuged (200 × *g*, 1 min), and the supernatant was subjected to RNA extraction. 200 μL nasopharyngeal swabs collected in commercial UTM™ kits were used for RNA extraction. Serum/plasma samples were collected from the peripheral blood obtained from patients, and 200 μL of each sample was used for RNA extraction. The viral RNA extraction was performed using a fully automated instrument (PCL, South Korea) with the Real-prep Viral DNA/RNA Kit (Biosewoom, South Korea).

3.4. Identification of SARS-CoV-2 infectious virus using cell culture

In order to identify the viable infectious SARS-CoV-2, Vero E6 cells lines (Cell Line Bank, KCLB no. 21587) were used for culturing and identification. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum in 6-well culture plates. The culture medium was supplemented with 1× penicillin–streptomycin antibiotic solution (Gibco, Thermo

Fisher Scientific Inc. Korea) substituted with a 5% atmospheric CO_2 at 37 °C. The swab samples collected in the UTM™ kit was further diluted with 1 mL of Dulbecco's phosphate-buffered saline (Welgene, Korea) and inoculated into cultured Vero cell monolayer. After two passages, viral proliferation was confirmed based on RT-qPCR with a confirmatory C_t value of < 20 or with indirect immunofluorescence assay (IFA) using in-house SARS-CoV-2 antigen slides. In addition the culture was examined daily for cytopathic effects, similarly to the procedures used for SARS-CoV and MERS-CoV in other studies [85, 86]. All cell culture infection experiments were performed at the Health and Environment Research Institute of Gwangju City and Chosun university hospital with a biosafety level-3 laboratory.

3.5. Indirect ELISA for antibody detection

An Indirect ELISA was performed for serological assays to find the antibody titers of IgG, IgM, and total antibody (IgG, IgM, and IgA), against the plant-expressed recombinant nucleoprotein of SARS-CoV-2. In brief, 100 µL of 2 µg/mL plant recombinant SARS-CoV-2 nucleocapsid protein (BIOAPP. Inc., Korea) were coated in each well of 96-well ELISA microplates (Thermo Fisher Scientific, Waltham, MA, USA.) with carbonate-bicarbonate buffer followed by overnight incubation at 4 °C. Then the plates were washed with PBS supplemented with 0.05% of Tween 20 (PBS-T) and blocked with blocking buffer (PBS-T containing 5% of skim milk) at 37 °C

for 2 h. After washing, a 100-fold dilution of serum samples in blocking buffer were added and further incubated for 2 h at 37 °C. The plates were rewashed, and a secondary antibody [a horseradish peroxidase–conjugated goat anti-human IgG antibody (1:6000 Invitrogen, Thermo Fisher Scientific, Cat A18805), an anti-human IgM antibody (1:3000 Invitrogen, Thermo Fisher Scientific, Cat 31415), or an anti–human-total-antibody antibody (1:40,000; Thermo Fisher Scientific, Cat 31418)] was added, and incubated for another 1 h at 37 °C. The plates were further washed extensive to remove all the unbound antibodies and 50 μL of the 3,3′5,5′-tetramethylbenzidine substrate (TMB, Sigma-Aldrich, St. Louis, MO, USA) was added into all well and incubated for 30 min in room temperature in dark. Then 25 μL of 1 M H_2SO_4 was added to stop the reaction, and the plates were measured at an optical density of 450 nm (OD_{450}). All ELISA experiment were performed as triplicate and assayed for the results. For the determination of cutoff values, the mean OD_{450} of negative serum samples plus threefold standard deviation of the samples were calculated. Hence, 1.1, 0.5, and 0.7 for IgG, IgM, and total antibody were observed as the cutoffs cutoff values. When the OD of a patient sample was greater than the calculated cutoff, it was considered positive for SARS-CoV-2.

3.6. Detection of N antigen for antigenemia using sandwich ELISA

For the identification of the SARS-CoV-2 nucleocapsid protein (*NP*) antigenemia assay in COVID 19 patients and negative subjects was performed using sandwich ELISA, where a single molecule array (SIMOA) technology with paramagnetic microbeads were utilized. SIMOA SARS-CoV-2 *NP* Protein Advantage kit assay (Quanterix Corp, Boston, MA, USA, PN/103806) was a digital immunoassay which quantitatively measure the nucleocapsid protein of SARS-CoV-2 in plasma and serum. Briefly, 96-well ELISA microplates (Quanterix® plates) was loaded with 4x dilution of serum or plasma and the assay was carried out in Simoa HD-X instrument (Quanterix) composed of two□step immunoassay. Furthermore, the assay incubation was performed simultaneously with target antibody coated with paramagnetic beads together with sample and biotinylated antibody of detection (SIMOA Guide Quanterix). Hence the nucleocapsid protein (antigenemia) present in the sample was captured by the antibody coated beads which bound along with biotinylated antibody, whereas the detection was simultaneously measured as described before [87, 88].

3.7. The Immunofluorescence assay for the detection of SARS-CoV-2

Vero E6 cell lines were infected with SARS-CoV-2 virus which was obtained from Korea Disease Control and Prevention Agency. The antigen slides of SARS-CoV-2 were prepared as follows, briefly, Vero E6 cell lines cultured on the Teflon-coated multiwell slides for three days were infected with the SARS-CoV-2 virus overnight at 37 °C with 5% CO_2. On the next day the slides were fixed with 80% acetone for the detection of fluorescence assay. In order to perform the IFA, patient's serum was diluted with twofold serial dilution starting from 1:16 and added with SARS-CoV-2 viral antigens slides, followed by incubation for 30 min at 37 °C in moist chamber. The slides were further washed and incubated with a 1:400-diluted secondary antibody (a fluorescein isothiocyanate–conjugated anti-human IgM or IgG antibody; (MP Biomedicals, OH, USA). Then, the slides were mounted after dispensing the mounting solution (VECTOR Laboratories) and observed under a fluorescence microscope (Olympus IX73, magnification: 400×). A cutoff value for the IFA was set by using the clinical samples obtained from 15 healthy individuals, for IgG antibody, titer ≥1:32 was chosen as the positive cutoff value [89].

3.8. One step reverse transcription quantitative real time PCR (RT-qPCR) for SARS-CoV-2 viral RNA detection

RT-qPCR was performed using various target genes which includes, the nucleocapsid protein (*NP-gene)*, envelope protein (*E-gene)*, and RNA dependent RNA polymerase (*RdRp-gene)* genes. The PCR was performed along with a reference gene as a positive control and an RNAase free distilled water as a negative control. The *NP* gene primers and probes were designed in-house (for iNP assay) and the primers and probe were, nCov-NP_572F (5′-GCAACAGTTCAAGAAATTC-3′) 1 µL (10 pmol/µL), nCov-NP_687R (5′-CTGGTTCAATCTGTCAAG-3′) 1 µL (10 pmol/µL), and nCov-NP_661P (5′-FAM-AAGCAAGAGCAGCATCACCG-BHQ1-3′) 1 µL (5 pmol/µL) and utilized for PCR. The RT-qPCR was standardized with the following conditions and performed using Exicycler™ 96 (Ver.4) Real-Time Quantitative Thermal Block (Bioneer, South Korea0. Briefly 4 µL of 5X RT-qPCR mixture (Roche), 0.5 µL of 200X RT enzyme solution (Roche), 5 µL of template and 7.9 µL of RNAase free water, which constitutes a 20 µL reaction. Thermal cycling was performed as follows: 50 °C for 10 min for reverse transcription, one cycle of 95 °C for 30 s for pre-incubation, 95 °C for 5 s at 57 °C for amplification, and 45 cycles for data detection. Furthermore, for *E* and *RdRp* target genes, the Kogene Kit (Kogene Biotech Co., Ltd., Seoul, South

Korea) and SD Kit (SD Biotechnologies Co., Ltd.,Seoul, South Korea) were utilized and the amplification was performed according to the manufacturer's specifications. Cycle threshold (C_t) values were set as ≤35 and ≤40 with respective of a reference gene and were assumed to denote the positive result of SARS-CoV-2. In addition, to compare the specificity and sensitivity of primer sets we use other neutral primers used around the globe, in this study we chose the primers recommended by the CDC and WHO. All primer details are listed in Table 1.

Table 1. All primers, probes, and PCR conditions used in this study.

Primer source	Target gene	Primer name	Primer sequence	Target	PCR condition
WHO primer	**RdRp gene**	RdRP_SARSr-F2	GTGARATGGTCATGTGTGGCGG	Pan Sarbeco	50℃ for 10 min, 1 cycle for Reverse transcription. 95℃ 30sec for 1 cycle for pre-incubation, 95℃ 5sec for and 57℃ for amplification and data detection for 45 cycles
		RdRP_SARSr-R1	CARATGTTAAASACACTATTAGCATA		
		RdRP_SARSr-P1	[TET]CCAGGTGGWACRTCATCMGGTGATGC[BHQ1]		
CDC primer	**N gene 1**	2019-nCoV_N1-F	GACCCCAAAATCAGCGAAAT	SARS-CoV2	
		2019-nCoV_N1-R	TCTGGTTACTGCCAGTTGAATCTG		
		2019-nCoV_N1-P	[FAM]ACCCCGCATTACGTTTGGTGGACC[BHQ1]		
CDC primer	**N gene 2**	2019-nCoV_N2-F	TTACAAACATTGGCCGCAAA		
		2019-nCoV_N2-R	GCGCGACATTCCGAAGAA		
		2019-nCoV_N2-P	[FAM]ACAATTTGCCCCCAGCGCTTCAG[BHQ1]		
CDC primer	**N gene 3**	2019-nCoV_N3-F	GGGAGCCTTGAATACACCAAAA		
		2019-nCoV_N3-R	TGTAGCACGATTGCAGCATTG		
		2019-nCoV_N3-P	[FAM]AYCACATTGGCACCCGCAATCCTG[BHQ1]		
This study (iNP)	**N gene**	nCov-NP_572F	GCAACAGTTCAAGAAATTC	SARS-CoV2	59℃ for amplification and the rest is same like above
		nCov-NP_687R	CTGGTTCAATCTGTCAAG		
		nCov-NP_661P	[FAM]AAGCAAGAGCAGCATCACCG[BHQ1]		
Kogene Kit	**E gene**	Primer/Probe mix 1 (E-gene)	Manufacturer's trade mark	SARS-CoV2	As per Manufacturer's instructions
	RdRp gene	Primer/Probe mix 2 (RdRp gene)	Manufacturer's trade mark	SARS-CoV2	As per Manufacturer's instructions

3.9. SARS-CoV-2 genome variant detection

Few samples were send to commercial sequencing for NGS, and the obtained sequences were deposited in GISAID. In addition to the NGS we utilized the commercial mutation identification kit (PowerChek™ SARS-CoV-2 S-gene Mutation Detection Kit) from Kogene biotech, Seoul, South Korea for variants and mutation identification. Furthermore, an in-house designed nested PCR targeting spike protein gene was performed using the following primer sets. For 1st PCR, (for 1st nCoV_S-635F 5′- TAGTGCGTGATCTCCCTC-3′, nCoV_S-2200R 5′-TCTTGGTCATAGACACTGG-3′ and for 2nd PCR, nCoV_S-860F 5′-CTGTAGACTGTGCACTTGAC-3′ nCoV_S-1980R 5′-GAGTTGTTGACATGTTCAGC3′) were used in this study. The obtained positive amplicons were directly sequenced commercially and the sequence obtained were analyzed using BLAST from NCBI (National Institutes of Health, Rockville, MD, USA). Furthermore the sequences were aligned to construction a phylogenetic tree for variant confirmation. To construct the phylogenetic tree for variant identification, ClustalX (version 2.0; www.clustal.org/) and Tree Explorer program (DNASTAR, Madison, WI) and Molecular Evolutionary Genetics Analysis (MEGA) software was used.

3.10. Classification of COVID-19 patients in accordance to Severity

In order to determine the diagnostic characteristics, the patients were categorized as asymptomatic, mild to moderate, severe, and critical or fatal according to the Sixth Revised Trial Version of the Novel Coronavirus Pneumonia Diagnosis and Treatment Guidance and as per the clinical parameters and guidelines set by CDC [90].

3.11. Statistical Methods

Sensitivity and specificity, as well as accuracy are expressed in percentages. Quantitative variables are presented as mean ± standard deviation for normally distributed variables or as the median (range) and a percentage (95% confidence interval). Means were compared using *t*-tests for continuous variables. Categorical variables were compared by either the chi-square or Fischer's exact test, when appropriate. Continuous variables were compared by the Mann–Whitney nonparametric test, when appropriate. The mean data were used to evaluate sensitivity and specificity via the area under the receiver operator characteristic (ROC) curve. Coefficients of determination (R^2) were computed using linear regression analysis, which was used for multiple correlation analysis. To determine the 30 or 40-days mortality rate or survival rate, Kaplan–Meier survival analysis was

performed in the RNAemia group, non-RNAemia group, and antigenemia and non-antigenemia groups. To investigate the correlation of predictive risk factors for the different variables, univariate and multiple logistic regression analysis were performed. Viral load comparisons were analyzed using the nonparametric Kruskal–Wallis test followed by the Mann–Whitney U test. Statistical significance was set at $P < 0.05$. All statistical analyses were performed using MedCalc 20.013 software (Ostend, Belgium), IBM SPSS Statistics for Windows, version 26.0. (IBM Corp., Armonk, NY, USA) and GraphPad Prism 9 (San Diego, CA, USA) [91].

IV. RESULTS

4.1. Diagnostic accuracy of SARS-CoV2 real-time polymerase chain reaction

4.1.1. Clinical characteristic of patients

For the study of diagnostic accuracy of RT-qPCR the patients were recruited between February 21 and May 11, 2020 during the first wave of SARS-CoV-2 infection in Korea. We enrolled 12 confirmed COVID-19 patients, whereas the median age of the patients were 49.16 years (range, 22–79 years), including 7 men and 5 women. Among all patients recruited, two patients were asymptomatic and did not possess any symptoms or clinical signs of COVID-19 during the entire period of this study analysis. In addition, to the confirmed patients we enrolled 128 SARS-CoV-2–negative samples collected form 107 healthy subjects as presented in Table 2. The clinical and subclinical features and finding of all COVID-19 patients, which includes, age, sex, signs and symptoms, along with serological assay were presented in Table 3.

Table 2. Diagnostic characteristics of negative control samples

Diagnostic characteristics	Negative samples			
	Nasopharynx	**Sputum**	**Oropharynx**	**Saliva**
No. of samples	55	21	24	28
NP-gene (iNP) RT-qPCR	ND	ND	ND	ND
E-gene (Kogene kit) RT-qPCR	ND	ND	ND	ND
RdRp-gene (Kogene kit) RT-qPCR	ND	ND	ND	ND
WHO RdRp Primers RT-qPCR	ND	ND	ND	ND
CDC N1 primers RT-qPCR	ND	ND	ND	ND
CDC N2 primers RT-qPCR	2 (+)	2 (+)	1 (+)	1 (+)
CDC N3 primers RT-qPCR	ND	ND	ND	ND

Fifty-five nasopharyngeal, 21 sputum, 24 oropharyngeal, and 28 saliva samples were obtained from health checkup subjects with no clinical symptoms, no increase in a SARS-CoV-2 antibody titer, or no contact with confirmed COVID-19 patients. Cycle threshold >35 indicates a negative result; ND: not detectable; 2(+): two SARS-CoV-2–positive samples; 1(+): one virus-positive sample

Table 3. Clinical and subclinical characteristics of all COVID-19 patients for accuracy of primers study.

Patient	Gender/ Age	Underlying Comorbidity	Symptoms & signs	IFA-Titer		ELISA-Titer			Cell culture
				IgG Initial/ Follow-up	IgM Initial/ Follow-up	IgM Initial/ Follow-up	IgG Initial/ Follow-up	Total antibody Initial/ Follow-up	
1	M/46	HTN, dyslipidemia	coughing, chill	<1:32/1:256	<1:32/<1:32	<1:128/1:512	<1:128/1:4096	<1:128/1:4096	(+)
2	M/30	HTN	coughing, sore throat chill	<1:32/1:128	<1:32/<1:32	<1:128/<1:128	<1:128/1:512	<1:128/1:512	(+)
3	F/79	HTN,DM, dyslipidemia, GBS	dyspnea	<1:32/1:1,024	<1:32/<1:32	<1:128/1:512	<1:128/1:2048	<1:128/1:2048	(+)
4	M/30	None	febrile sense	<1:32/1:128	<1:32/<1:32	<1:128/1:128	<1:128/1:1024	<1:128/1:1024	(+)
5	F/29	None	sore throat, myalgia chill	<1:32/1:64	<1:32/<1:32	<1:128/<1:128	<1:128/1:128	<1:128/<1:128	(+)
6	M/74	HTN,DM, dyslipidemia	sore throat, rhinorrhea	1:256/1:1,024	<1:32/<1:32	1:128/1:256	1:512/1:8192	1:512/1:4096	(-)
7	F/75	HTN, dyslipidemia	fever, sore throat	<1:32/>1:1,024	<1:32/1:32	<1:128/1:128	<1:128/1:1024	<1:128/1:1024	(-)
8	M/79	HTN,DM	fever, headache	<1:32/1:128	<1:32/<1:32	<1:128/1:256	<1:128/1:8192	<1:128/1:4096	(-)
9	F/61	None	fever, coughing	<1:32/1:256	<1:32/<1:32	<1:128/1:1024	1:256/1:16384	1:128/1:8192	(-)
10	M/36	None	sore throat, headache chill	<1:32/1:128	<1:32/<1:32	<1:128/<1:128	<1:128/1:128	<1:128/1:128	(-)
11	F/29	None	No symptoms	1:64/1:64	<1:32/<1:32	<1:128/<1:128	<1:128/1:128	<1:128/<1:128	(-)
12	M/22	None	No symptoms	1:64/1:64	<1:32/<1:32	<1:128/<1:128	<1:128/1:128	<1:128/1:128	(-)

HTN, hypertension; DM, diabetes mellitus, GBS; Guillain-Barre syndrome. The IFA titer was measured by serial dilution of a patient's serum, and the IgG titer cutoff was ≥1:32. Antibody titer detection was performed (with serial dilution) by an indirect ELISA at OD_{450} against a recombinant SARS-CoV-2 nucleoprotein; the cutoff titer for IgM was 0.5; for IgG, it was 1.1; and for total Ig, it was 0.7

4.1.2. Assessment of cross-reactivity of other respiratory viruses and bacteria using *in vitro* RT-qPCR analysis

Various kinds of respiratory-disease bacterial strains and viruses were assayed using RT-qPCRs with all the primers utilized in this study. The Kogene Kit, *E* gene primer set, WHO *RdRp* primer set, and the CDC *N2* and *N3* primer set showed cross-reactivity with the purified SARS-CoV urbani strain. Similarly the WHO, *RdRp* primer set had a cross reactivity with *Influenza A* virus with a higher C_t value. Whereas the CDC *NI* and *N2* primer sets had cross reactivity with *Influenza A, Influenza B, Influenza C virus with* C_t value above 35. However, the reliability of the CDC *N1* primer set varied from other primer sets when the C_t value was set above >35. Our study data suggested that RT-qPCR with the iNP primer set and *RdRp* primer set of Kogene Kit has better specificity for the SARS-CoV-2 virus as summarized in table 4.

Table 4. RT-qPCR results on other respiratory viruses and bacteria.

Type	Virus/Bacteria strain name	RT-qPCR results of different target genes and primer sets (Cycle threshold)						
		NP-gene (iNP)	E-gene (Kogene kit)	RdRp-gene (Kogene kit)	WHO RdRp Primers	CDC N1 primers	CDC N2 primers	CDC N3 primers
Virus	Avian infectious bronchitis virus, strain *Massachusetts*	ND	ND	ND	ND	35.84	ND	ND
Virus	Human *Coronavirus* NL63	ND	ND	ND	ND	38.47	ND	ND
Virus	*Canine coronavirus* Strain UCD1	ND	ND	ND	ND	37.04	ND	ND
Virus	*MERS-CoV*	ND	ND	ND	UD	ND	ND	ND
Virus	*SARS-CoV* Purified, in PBS 1X10^8 pfu/ml (eq), Urbani strain	ND	24.23	ND	31.76	38.08	30.10	25.36
Virus	*Human respiratory syncytial virus*, Strain A2000/3-4	ND	ND	ND	ND	36.47	38.95	ND
Virus	*Influenza A/Texas/36/91, H1N1*	ND	ND	ND	35.51	37.55	38.57	ND
Virus	*Influenza B/Florida/4/2006*	ND	ND	ND	ND	ND	37.81	ND
Virus	*Influenza C virus C/Taylor/1233/1947*	ND	ND	ND	ND	39.17	39.03	ND
Virus	*Measles virus* Edmonston	ND	ND	ND	ND	ND	39.02	ND
Virus	*Rhinovirus*	ND	ND	ND	ND	ND	ND	ND
Virus	Human astrovirus (HAstV) type 1	ND	ND	ND	ND	ND	ND	ND
Virus	Human astrovirus (HAstV) type 2	ND	ND	ND	ND	36.34	ND	ND
Bacteria	*Klebsiella pneumoniae* Isolate 1	ND	ND	ND	ND	38.70	ND	ND
Bacteria	*Klebsiella oxytoca* MIT 10-5244	ND	ND	ND	ND	37.16	ND	ND
Bacteria	*Leptospira interrogans* -- HAI0156 (Serovar Copenhageni)	ND	ND	ND	ND	37.13	ND	ND
Bacteria	*Mycobacterium abscessus* #103	ND	ND	ND	ND	37.37	ND	ND
Bacteria	*Mycobacterium avium* 2285 Smooth	ND	ND	ND	ND	37.10	ND	ND
Bacteria	*Mycobacterium intracellulare* 1956	ND	ND	ND	ND	37.30	ND	ND
Bacteria	*Staphylococcus aureus* Strain AIS 1000505 AKA VRS10	ND	ND	ND	ND	37.73	ND	ND
Bacteria	*Staphylococcus; aureus* MRSA; M0200	ND	ND	ND	ND	37.02	ND	ND
Bacteria	*Streptococcus pneumoniae* Strain TCH8431	ND	ND	ND	ND	ND	ND	ND
Bacteria	*Pseudomonas aeruginosa* ATCC 27853	ND	ND	ND	ND	ND	ND	ND

ND: not detectable; cycle threshold values are presented as obtained here. Cycle threshold >35 is considered a negative result for the Kogene Kit; iNP: the in-house–designed primer set targeting the *NP gene*

4.1.3. Evaluation of SARS-CoV-2 via RT-qPCR

From the 12 patients a total of 590 various clinical samples, including nasopharynx (17.1%), oropharynx (15.21%), sputum (17.9%), saliva (11.6%), urine (16.6%), stool (9.3%), serum/plasma, and whole blood (13.05%) samples were collected and analyzed for the presence of SARS-CoV-2 viral RNA. For all patients, the viral load in the samples from symptoms onset to the recovery were analyzed. The earliest sample was collected 2 days prior to symptom onset, and the latest sample was taken on the 74th day post-recovery. Viral shedding from nasopharyngeal samples was substantially higher than that from oropharyngeal samples during the early stage of symptoms, from day 0 to day 7 (P = 0.006). Viral RNA could be detected up to day 14–15 in nasopharyngeal samples but for only day 7–8 in the oropharyngeal samples at a C_t cutoff of 35 (C_t-35). In sputum samples, we consistently detected viral RNA with low C_t values.

Simultaneously, RT-qPCR with the iNP primer set was carried out for all the samples collected in this study. In agreement with the previously published data [92] we were able to detect SARS-CoV-2 RNA in only 5 (6.32%) samples out of the 77 blood samples in the entire study period. Of the 101 nasopharyngeal and 90 oropharyngeal samples collected, 42 (44.68%) and 21 (23.59%) samples tested positive for SARS-CoV-2 with iNP RT-qPCR, respectively. Among the 106 sputum samples, 61 (57.54%) samples tested positive for this virus. To identify the presence

of viral RNA in saliva, 69 samples were collected, and 32 (46.37%) of these tested positive. Only 1 (1.08%) urine sample tested positive from the 92 urine samples collected, and only 6 (10.90%) out of 55 stool samples tested positive for SARS-CoV-2 with iNP RT-qPCR at C_t-35. The results are summarized in Table 5.

Table 5. RT-qPCR results on SARS-CoV-2 in all clinical samples for primer set iNP and primer sets targeting the *E* and *RdRp* genes (Kogene Kit)

	Specimens and values	Nasopharynx (N=101)	Oropharynx (N=90)	Sputum (N=106)	Pla/Ser/WB (N=77)	Saliva (N=69)	Stool (N=55)	Urine (N=92)
NP-gene (iNP)	Positive test results, No%	42 (44.68%)	21 (23.59%)	61 (57.54%)	5 (6.32%)	32(46.37%)	6 (10.90%)	1 (1.08%)
	Cycle threshold, Mean (SD)	26.13 (13.5)	29.39 (4.4)	27.26 (5.1)	30.39 (3.01)	28.47 (4.1)	29.62 (3.5)	NA
	Ct range	10.04 - 34.94	14.94 - 33.96	14.94 -33.96	27.49 - 33.91	21.60 – 34.69	25.34 – 34.15	NA
	Sensitivity/Specificity	43.1%/100%	24.3%/100%	58.1%/100%		43.3%/100%		
	95% CI for sensitivity	0.628 - 0.779	14.77 - 32.95	0.709 - 0.841	2.56 - 14.08	0.644 - 0.808	3.64 - 20 .0	NA
	Specimens and values	**Nasopharynx (N=97)**		**Sputum (N=104)**				
E-gene (Kogene kit)	Positive test results, No%	38 (39.17%)		49 (47.11%)				
	Cycle threshold, mean (SD)	25.39 (5.2)		27.83 (5.0)				
	Ct range	12.88 – 34.33		13.76 - 34.93				
	Sensitivity/Specificity	36.5%/100%		46.1%/100%				
	95% CI for sensitivity	0.593 - 0.750		0.638 - 0.784				
	Specimens and values	**Nasopharynx (N=97)**		**Sputum (N=104)**				
RdRp-gene (Kogene kit)	Positive test results, No%	37 (38.18%)		47 (45.19%)				
	Cycle threshold, Mean (SD)	25.62(5.2)		27.96 (5.0)				
	Ct range	13.40 - 33.68		14.17 – 34.68				
	Sensitivity/Specificity	36.1%/100%		46.6%/100%				
	95% CI for sensitivity	0.593 - 0.750		0.652 - 0.796				

RT-qPCR results on SARS-CoV-2 in all clinical samples collected from symptom onset to post-recovery, up to 5 weeks or more. Pla/Ser/WB: plasma/serum/whole blood; NA: not applicable; *N*: number; SD: standard deviation; CI: 95% confidence interval

In order to check the accuracy of iNP RT-qPCR, we cross-checked all the samples with the Kogene Kit targeting the *E* gene and the *RdRp* gene. For rigorous quality standards and cost effectiveness, we selected only nasopharyngeal and sputum samples for the evaluation of the specificity and sensitivity of other primer sets such as the WHO primers, CDC primers, and Kogene Kit.

4.1.4. Diagnostic accuracy of samples up to 3 days after admission along with comparison with various RT-qPCR primer and probe sets

To scrutinize the diagnostic specificity and sensitivity and to examine all the primer sets targeting SARS-CoV-2, we selected the nasopharyngeal and sputum samples collected between days 0 to 3 after hospital admission. Meanwhile, we attempted to determine differences in cutoffs values, wherein we chose 35 and 40 as the cutoff for all RT-qPCR primers. When the cutoff C_t-35 was used, the sensitivity/specificity of iNP RT-qPCR in sputum was 94.8%/100%, and in nasopharyngeal samples was 69.6%/100%, respectively. RT-qPCRs with the *E* gene and *RdRp* gene primer sets (Kogene Kit) had sensitivity and specificity of 84.2%/100% and 94.8%/100% in sputum samples and 60.9%/100% and 60.9%/100% in nasopharyngeal samples, respectively (Table 6).

Table 6. Sensitivity and specificity of RT-qPCR of samples from the day of admission to the 3rd day, for various primers and probes

Primer sets	Sputum samples		Nasopharynx samples	
	Ct-35	Ct-40	Ct-35	Ct-40
NP-gene (iNP)	94.8%/100%	100% /100%	69.6% /100%	73.9% /100%
Sensitivity/Specificity (AUC)	(0.97)	(1.0)	(0.82)	(0.87)
E-gene (Kogene kit)	84.2% /100%	89.5% /100%	60.9% /100%	60.9% /100%
Sensitivity/Specificity (AUC)	(0.92)	(0.95)	(0.80)	(0.80)
RdRp-gene (Kogene kit)	94.8%/100%	100% /100%	60.9% /100%	65.2% /100%
Sensitivity/Specificity (AUC)	(0.97)	(1.0)	(0.83)	(0.79)
WHO RdRp Primers	42.1%/100%	79.0%/100%	65.2% /100%	86.4% /96.4%
Sensitivity/Specificity (AUC)	(0.71)	(0.90)	(0.82)	(0.90)
CDC N1 primers	57.9%/100%	89.5%/90.5%	69.6% /100%	82.6% /85.7%
Sensitivity/Specificity (AUC)	(0.79)	(0.86)	(0.85)	(0.84)
CDC N2 primers	63.2%/90.5%	73.7%/100%	65.2% /96.4%	60.3% /96.4%
Sensitivity/Specificity (AUC)	(0.73)	(0.79)	(0.79)	(0.67)
CDC N3 primers	57.9%/100%	89.5%/100%	69.6% /100%	73.9/100%
Sensitivity/Specificity (AUC)	(0.79)	(0.95)	(0.85)	(0.87)

Number of SARS-CoV-2–positive sputum samples: 19; number of virus-negative sputum samples: 21; number of virus-positive nasopharyngeal samples: 23; number of virus-negative nasopharyngeal samples: 28; **iNP**: in-house–designed *NP* gene primer set; **C_t-35**: cutoff cycle threshold of 35; **C_t-40**: cutoff cycle threshold of 40

In contrast, RT-qPCR with the WHO RdRp primer set manifested a sensitivity/specificity of 42.1%/100% in sputum samples and 65.2%/100%, in nasopharyngeal samples. On the other hand, the RT-qPCRs with CDC *N1*, *N2*, and *N3* primers had a sensitivity/specificity of 69.6%/100%, 65.2%/96.4%, and 69.6%/100% in nasopharyngeal samples and 57.9%/100%, 63.2%/90.5%, and 57.9%/100% in sputum samples, respectively, as summarized in Table 6.

When C_t was set to 40, a slight increase in sensitivity was observed in RT-qPCR involving either the iNP primers or Kogene Kit primers, whereas RT-qPCR with the primers recommended by the WHO and CDC showed significantly varied specificity and sensitivity (Figure 4).

In brief, RT-qPCR using the primers, either iNP or the *RdRp* gene (Kogene Kit) had the highest sensitivity (94.8%, C_t-35) in the sputum samples as compared with all the other primers. Nevertheless, in the nasopharyngeal samples, the sensitivity of iNP at C_t-35 was 69.9%, and the sensitivity of the CDC primers targeting genes *N1* (69.6%), *N2* (65.2%), and *N3* (69.6%) was comparable, as presented in Table 6. All primer sets were 100% specific in both the sputum and nasopharyngeal samples, with the exception of the *N2* gene (90.5% specificity in sputum and 96.4% in nasopharynx samples), respectively. Hence, RT-qPCR with the iNP primers was superior to RT-qPCR involving the (target gene *E*) Kogene Kit primers (iNP vs. *E* gene: $P = 0.014$ in sputum, and iNP vs. *E* gene: $P = 0.056$ in nasopharyngeal samples)

when considering sensitivity in both sputum and nasopharyngeal samples (Table 6). Our results suggested that for the detection of SARS-CoV-2, sensitivity in sputum samples is significantly higher than that in nasopharyngeal samples and is more suitable for PCR-based diagnosis (C_t-35, $P = 0.0193$; C_t-40, $P = 0.0012$; Figure 5a, b).

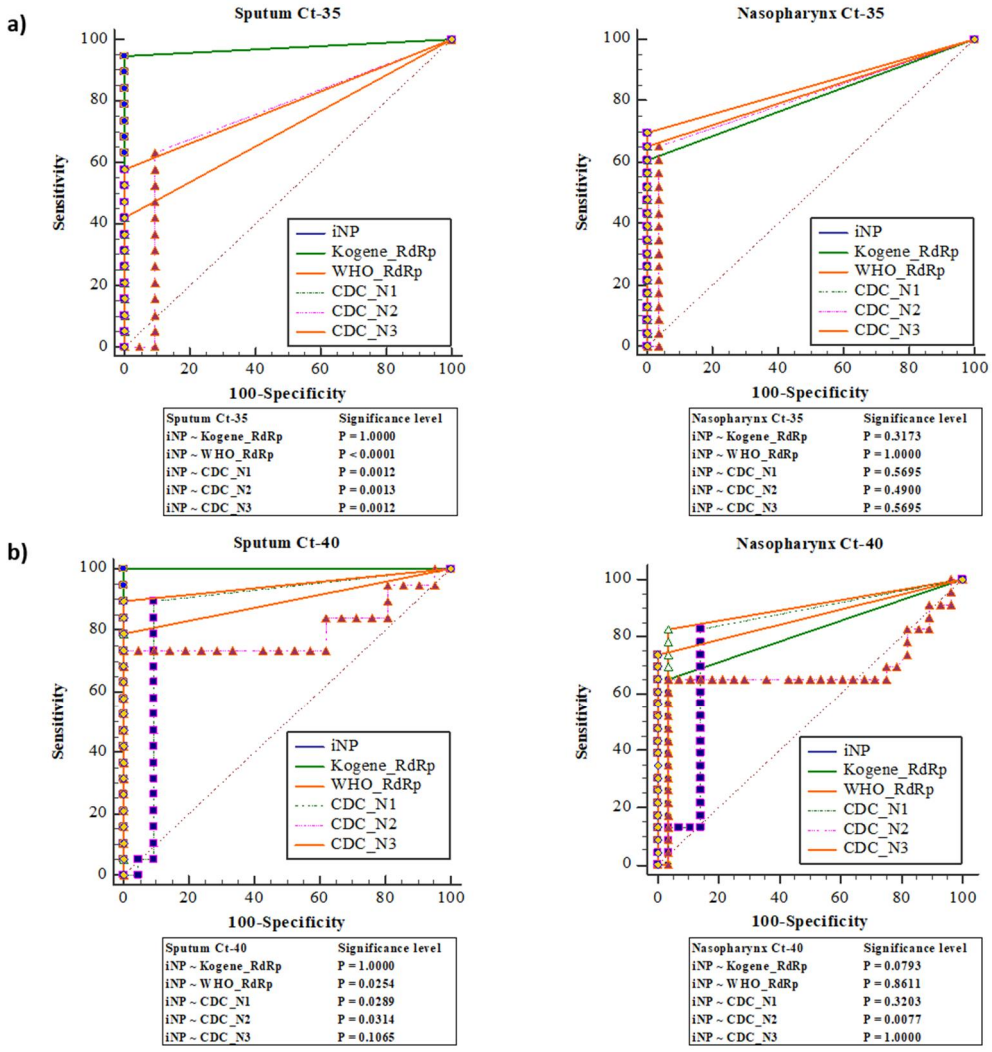


Figure 4: Comparison of the specificity and sensitivity of various commercial RT-qPCR primer sets among selected clinical samples. a) Evaluation of specificity and sensitivity in sputum and nasopharyngeal samples at a C_t cutoff of 35 (C_t-35). b) Evaluation of specificity and sensitivity in sputum and nasopharyngeal samples at a C_t cutoff of 40 (C_t-40).

4.1.5. Analysis of the specificity and sensitivity of RT-qPCR depending on the duration of COVID-19

In order to determine changes with time, we further analyzed specificity and sensitivity in the samples collected from the patients depending on the time interval from the date of admission to complete recovery. The samples at different time points from the date of admission, namely, at 0–3 days, 1 week, 2 weeks, 3 weeks, 4 weeks, and 5 or more weeks, were analyzed for specificity and sensitivity (Table 7).

To compare the results, we examined specificity and sensitivity at both C_t-35 and C_t-40. As expected, the sensitivity of iNP RT-qPCR was significantly higher than that of the primers targeting *E* and *RdRp* genes with the Kogene Kit when the C_t cutoff was 35. Meanwhile, the positivity rate of sputum samples was much higher than that of nasopharyngeal samples, as illustrated in Table 7. In the analysis of sensitivity depending on C_t, the sensitivity of iNP RT-qPCR at C_t-35 was significantly higher than that of the Kogene Kit. On the other hand, when the C_t cutoff was 40, the sensitivity markedly increased for both target genes *E* and *RdRp* of the Kogene Kit. This finding may be due to nonspecific bands similar to those reported in another study [93], because the manufacturer (Kogene) recommends a C_t cutoff of 35, our hypothesis of nonspecificity may be valid (Figure 4 and Table 7).

Table 7. RT-qPCR specificity and sensitivity in sputum versus nasopharyngeal samples from symptom onset to recovery

Days Since Symptoms Sensitivity (%)/Specificity (%) (AUC)	Sputum (N=101)						Nasopharynx (N=106)					
	Ct-35			Ct-40			Ct-35			Ct-40		
	Design	Kogene Kit		Design	Kogene Kit		Design	Kogene Kit		Design	Kogene Kit	
	NP gene	E gene	RdRP	NP gene	E gene	RdRP	NP gene	E gene	RdRP	NP gene	E gene	RdRP
Admission date	80.0/100 (0.90)	70.0/100 (0.85)	70.0/100 (0.85)	100/100 (1.0)	90.0/100 (0.95)	90.0/100 (0.95)	66.7/100 (0.83)	66.7/100 (0.83)	66.7/100 (0.83)	66.7/100 (0.83)	83.3/100 (0.92)	75.0/100 (0.87)
0 – 3 days	86.4/100 (0.93)	81.8/100 (0.91)	81.8/100 (0.91)	90.9/100 (0.95)	95.4/100 (0.98)	95.4/100 (0.98)	65.0/100 (0.83)	70.0/100 (0.85)	65.0/100 (0.83)	65.0/100 (0.83)	75.0/100 (0.86)	70.0/100 (0.85)
1 week (0 - 7 days)	83.9/100 (0.92)	77.4/100 (0.89)	80.6/100 (0.90)	87.1/100 (0.93)	90.3/100 (0.95)	90.3/100 (0.95)	73.5/100 (0.87)	70.6/100 (0.85)	70.6/100 (0.85)	79.4/100 (0.90)	79.4/100 (0.90)	76.5/100 (0.88)
2 weeks (8 – 14 days)	72.0/100 (0.86)	52.0/100 (0.76)	52.0/100 (0.76)	84.0/100 (0.92)	72.0/100 (0.86)	72.0/100 (0.86)	47.8/100 (0.74)	34.8/100 (0.67)	34.8/100 (0.67)	52.2/100 (0.76)	52.2/100 (0.76)	52.2/100 (0.76)
3 weeks (15 – 21 days)	42.1/100 (0.71)	21.1/100 (0.60)	21.1/100 (0.60)	47.4/100 (0.74)	36.8/100 (0.68)	47.4/100 (0.74)	17.7/100 (0.59)	5.9/100 (0.53)	5.9/100 (0.53)	23.5/100 (0.62)	29.4/100 (0.65)	23.5/100 (0.62)
4 weeks (22 – 29 days)	50.0/100 (0.75)	50.0/100 (0.75)	33.3/100 (0.67)	83.3/100 (0.92)	50.0/100 (0.75)	50.0/100 (0.75)	0/100 (0.50)	0/100 (0.50)	0/100 (0.50)	0/100 (0.50)	0/100 (0.50)	0/100 (0.50)
5 weeks+ (30 + days)	15.8/100 (0.58)	5.3/100 (0.53)	5.3/100 (0.53)	42.1/100 (0.71)	26.3/100 (0.63)	15.8/100 (0.58)	7.1/100 (0.54)	0/100 (0.50)	0/100 (0.50)	21.4/100 (0.61)	7.1/100 (0.54)	14.3/100 (0.57)

Samples were segregated as follows: admission date, 0–3 days after admission, 0–7 days after admission as 1 week, 8–14 days as 2 weeks, 15–21 days as 3 weeks, 22–29 days as 4 weeks, and 30+ days as 5 weeks+. *N*, number of samples; C_t-35, cycle threshold (cutoff) of 35; C_t-40, cycle threshold (cutoff) of 40. All samples were collected between days 0 and 3 after symptom onset

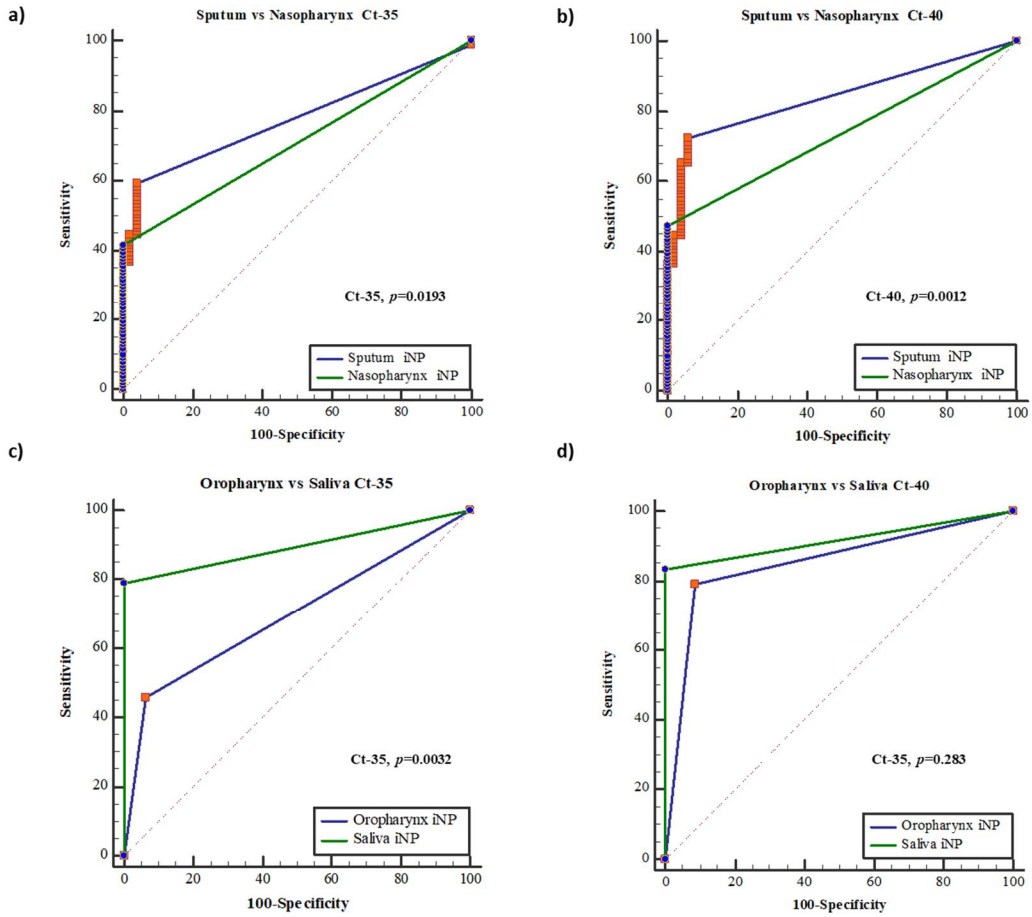


Figure 5: The specificity and sensitivity of RT-qPCR with the in-house–designed *NP* gene primer set (iNP) in nasopharyngeal vs. sputum samples and in oropharyngeal vs. saliva samples. a) Determination of specificity and sensitivity in selected sputum vs. nasopharyngeal samples at a C_t cutoff of 35 (C_t-35). b) Determination of specificity and sensitivity in selected sputum vs. nasopharyngeal samples at a C_t cutoff of 40 (C_t-40). c) Determination of specificity and sensitivity in selected (first week) oropharyngeal vs. saliva samples at a C_t cutoff of 35 (C_t-35). d) Determination of specificity and sensitivity in selected (first week) oropharyngeal vs. saliva samples at a C_t cutoff of 40 (C_t-40).

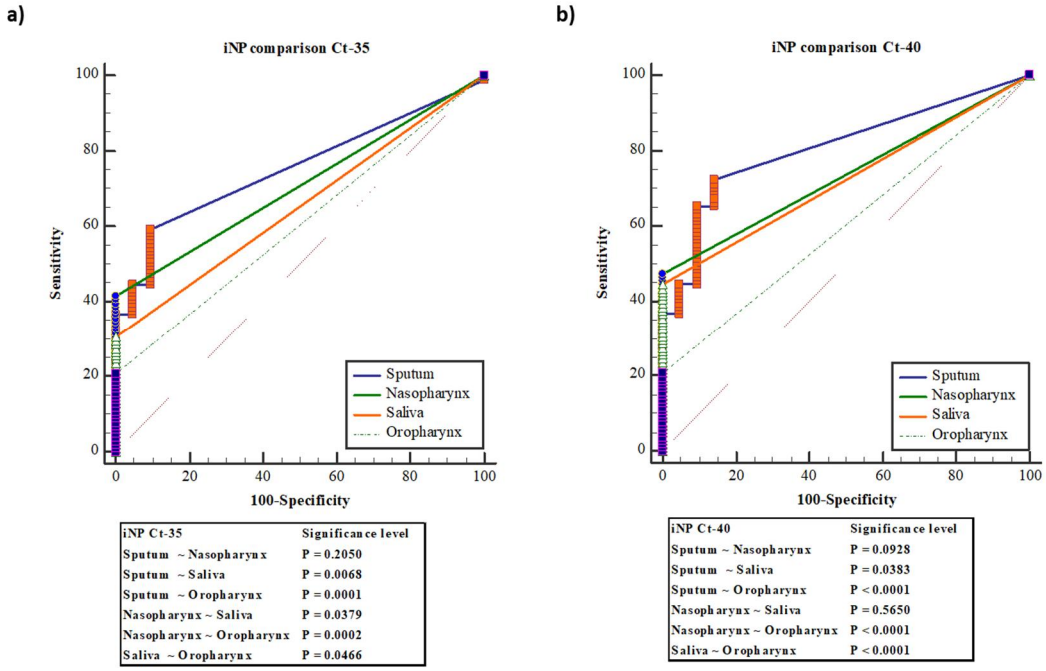


Figure 6: Comparison of the specificity and sensitivity of RT-qPCR involving the in-house–designed *NP* gene primer set (iNP) among sputum, nasopharyngeal, saliva, and oropharyngeal samples. a) Determination and comparison of the specificity and sensitivity of iNP RT-qPCR among sputum, nasopharyngeal, saliva, and oropharyngeal samples at a C_t cutoff of 35 (C_t-35). b) Determination and comparison of the specificity and sensitivity of iNP RT-qPCR among sputum, nasopharyngeal, saliva, and oropharyngeal samples at a C_t cutoff of 40 (C_t-40).

4.1.6. Comparison of the specificity and sensitivity of iNP RT-qPCR among sputum, nasopharyngeal, saliva, and oropharyngeal samples

We analyzed and compared the specificity and sensitivity of iNP RT-qPCR among various samples (nasopharyngeal, oropharyngeal, and saliva samples) at C_t cutoffs of 35 or 40. A more significant difference was observed between the saliva and nasopharyngeal (P = 0.0379, Figure 6a) samples. Meanwhile, sensitivity in saliva was significantly higher than the oropharyngeal samples at C_t-35 (P = 0.0032) during the first week after symptom onset (Figure 5c, d). Our results indicated that in sputum samples, sensitivity is the highest, followed by nasopharyngeal, saliva, and oropharyngeal samples, as illustrated in Figure 6a and 6b. Thus, sputum samples can be considered the primary clinical material for COVID-19 diagnosis. Unfortunately, we cannot eliminate the risk of aerosolization of virus particles while collecting the sputum sample.

4.2. SARS-CoV-2 RNAemia as a prognostic factor of disease severity on COVID-19 patients

4.2.1. Clinical characteristics COVID-19 patients in RNAemia assay

For the study of RNAemia we recruited 95 patients with clinically confirmed COVID-19, admitted and treated at Chosun University Hospital, Gwangju, South Korea between February 2020 and May 2021. The median age of the patients were 64 ± 18·7 years, and the percentages of men and women were 48% and 52%, respectively. Moreover, among patients with underlying comorbidities (54%), 40% had hypertension and 23% had diabetes mellitus. The detailed characteristics are presented in Table 8. On admission, several patients had symptoms such as fever (18%), cough (17%), headache (5%), chills (13%), sore throat (7%), and myalgia (11%). Additionally, considering the treatment scenario, 51% of patients underwent supplemental oxygen with 24% with high oxygen flow, whereas 15% required mechanical ventilation. Approximately 44% of the patients received antiviral treatment, and 31% patients underwent steroid therapy concurrently (Table 8).

Table 8. Clinical characteristics of patients with COVID-19

Characteristics	Total (n = 95)	RNAemia presence (n = 19)	RNAemia absence (n = 76)	*P*-value
Male, N (%)	46 (48%)	9 (47%)	37 (49%)	0·918
Age, Mean ± SD	64±18.7	76·8±12	60·8±18.8	<0·001
Comorbidities, N (%)	51 (54%)	13 (68%)	38 (50%)	0·150
Cardiovascular disease, N (%)	15 (16%)	3 (16%)	12 (16%)	1·000
Diabetes mellitus, N (%)	22 (23%)	7 (37%)	15 (20%)	0·134
Hypertension, N (%)	38 (40%)	11 (58%)	27 (36%)	0·075
Chronic lung disease, N (%)	1 (1%)	1 (5%)	0 (0)	0·200
Cancer, N (%)	8 (8%)	1 (5%)	7 (9%)	0·579
Chronic kidney disease, N (%)	2 (2%)	1 (5%)	1 (1%)	0·362
Symptoms, N (%)				
Fever, N (%)	17 (18%)	6 (32%)	11 (15%)	0·082
Cough, N (%)	16 (17%)	3 (16%)	13 (17%)	0·891
Headache, N (%)	5 (5%)	1 (5%)	4 (5%)	1·000
Chill, N (%)	12 (13%)	2 (11%)	10 (13%)	0·757
Sore throat, N (%)	7 (7%)	1(5%)	6 (8%)	0·695
Myalgia, N (%)	10 (11%)	2 (11%)	8 (11%)	1·000
Treatments				
Supplemental oxygen, N (%)	48 (51%)	19 (100%)	28 (37%)	<0·001
High flow oxygen therapy, N (%)	23 (24%)	13 (68%)	10 (13%)	<0·001
Mechanical ventilation, N (%)	14 (15%)	11 (58%)	3 (4%)	<0·001
Antiviral, N (%)	42 (44%)	19 (100%)	23 (30%)	<0·001
Steroids, N (%)	29 (31%)	13 (68%)	16 (21%)	<0·001

Data are expressed as the mean ± SD or N (%). The data consisted of 95 patients who participated in the study. N: number of patients; SD: standard deviation

4.2.2. Laboratory and biochemical characteristics of the patients

To determine the diagnostic characteristics and the presence of viral RNAemia, the patients were categorized as asymptomatic, mild to moderate, severe, and critical or fatal according to the Sixth Revised Trial Version of the Novel Coronavirus Pneumonia Diagnosis and Treatment Guidance [90]. The percentages of white blood cells in severe and critical or fatal cases were elevated along with other biochemical features. In contrast, the lymphocyte count decreased in severe and critical or fatal cases, as presented in Table 9.

Table 9. Laboratory findings of patients with COVID-19 on admission

Laboratory Variables	Total (N = 95)	Asymptomatic (N = 9)	Mild to Moderate (N = 60)	Severe (N = 8)	Critical or Fatal (N = 18)
WBC × 10^9 per L	6·18±3·08	6·74±2·63	5·44±1·66	7·24±3·53	7·92±5·34
Neutrophils (%)	69·01±14·44	55·99±15·47	65·12±11·85	81·2±7·9	83·06±10·26
Lymphocytes (%)	22·33±12·07	35·12±13·62	24·96±10·34	12·14±5·08	11·71±7·57
Creatinine (mg/dL)	1·00±1·36	0·68±0·10	0·98±1·36	1·78±2·82	0·89±0·45
CRP (mg/dL)	4·47±6·09	0·28±0·33	2·18±3·00	6·19±5·80	11·48±7·88
Procalcitonin (ng/mL)	0·234±0·684	0·030±0·011	0·090±0·108	0·125±0·061	0·873±1·435
Troponin-I (ng/mL)	0·031±0·085	0·001±0·001	0·029±0·093	0·012±0·010	0·056±0·092
AST (U/L)	35·57±26·44	17·49±3·81	31·31±20·17	51·95±50·62	51·52±27·54
ALT (U/L)	25·78±32·21	16·36±7·19	24·91±30·36	51·43±70·78	22·01±8·77

Data are expressed as mean ±SD or N (%). N, number of patients; CRP, C-reactive protein; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

4.2.3. RNAemia-positive serum/plasma cell culture

Cell culture viral proliferation results were monitored using the supernatant of infected Vero E6 cell lines after two passages at an interval of 5 days via RT-qPCR with a confirmatory C_t value of < 20 or IFA. None of the 12 patients with RNAemia had positive culture results.

4.2.4. Assessment of viral RNAemia in serum/plasma samples

Serum/plasma samples were analyzed for the presence of the viral RNA of SARS-CoV-2 using RT-qPCR with the *NP* target gene. Viral RNAemia was not detected in asymptomatic patients during the entire study period. In the mild-to-moderate category, RNAemia was detected in the first day hospital admission samples (2%) and in the week 1 hospital admission samples (6%). The proportion of RNAemia in severely ill patients was 13% in both the on admission and week 1 samples; however, no RNAemia was detected in week 2 samples collected after hospitalization. In contrast, a substantial proportion of RNAemia was detected in critically ill or fatal patients with COVID-19 (67% of on admission samples), and the viral RNA decreased to 18% in the week 1 (Figure. 7). However, none of the samples from week 2 exhibited RNAemia. None of the 12 patients with RNAemia had positive cell culture results. The association according to disease severity was significant on admission ($x^2(3) = 48.376$, $P < 0.001$).

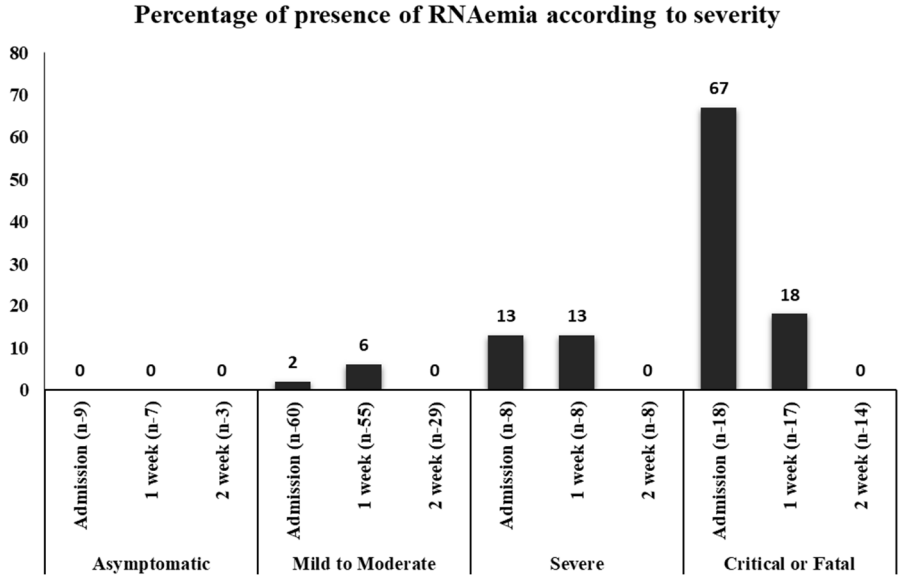


Figure 7: Direct proportion of viral RNAemia in disease severity. The plasma samples were classified according to the Sixth Revised Trial Version of the Novel Coronavirus Pneumonia Diagnosis and Treatment Guidance, and the data were expressed as N (%).

Hence, the proportion of viral RNAemia was directly correlated with disease severity (Table 10, Figure. 7). Similarly, the viral loads of plasma samples of critical or fatal cases were substantially higher in both on admission and week 1 samples than in the other groups of patients. Moreover, statistical analysis was performed for the viral load of RNAemia in different patient categories, and the results were significant for on admission samples, as listed in Table 10.

Table 10. Viral RNA presence in serum/plasma of patients with COVID-19

	Severity	RNAemia*			Viral load Mean (SD)		
		N (%)	Chi-Square	*P*-value		Kruskal–Wallis test *P*-value	Mann–Whitney U test *P*-value
Admission	Asymptomatic[a] (N = 9)	0 (0)	48·376[a]	<0·001	0	<0·001	<0·001[b,d] / 0·002[a,d] / 0·009[c,d]
	Mild to Moderate[b] (N = 60)	1 (2)			5·91E+01 (±4·58E+02)		
	Severe[c] (N = 8)	1 (13)			1·29E+02 (±3·64E+02)		
	Critical or Fatal[d] (N = 18)	12 (67)			6·62E+03 (±1·54E+04)		
Week 1	Asymptomatic (N = 7)	0 (0)	3·115[a]	0·374	0	0·360	
	Mild to Moderate (N = 51)	3 (6)			1·03E+02 (±4·23E+02)		
	Severe (N = 8)	1 (13)			1·22E+02 (±3·45E+02)		
	Critical or Fatal (N = 17)	3 (18)			5·24E+02 (±1·26E+03)		
Week 2	Asymptomatic (N = 3)	0 (0)	NA		0	NA	
	Mild to Moderate (N = 28)	0 (0)			0		
	Severe (N = 8)	0 (0)			0		
	Critical or Fatal (N = 14)	0 (0)			0		

Data are expressed as the mean ± standard deviation (SD) or N (%). N: number of patients; NA: not applicable. First sample is on admission, 1st week follow-up sample collected from the 5th to 9th day of admission, 2nd week sample collected from the 12th to 16th day of admission. * RNAemia comparisons among multiple subgroups were performed using the chi-square test. [a] represents the cells with the expected count. Viral load comparisons were analyzed using the chi-square test, followed by the nonparametric Kruskal–Wallis test and Mann–Whitney U test. [a, b, c,] and [d] represent the patient categories. $P < 0.05$ is considered significant.

4.2.5. Correlation of RNAemia with upper and lower respiratory tract specimens

Examination of the correlation of RNAemia with viral copy number in the upper and lower respiratory tract specimens showed that the levels of SARS-CoV-2 viral

loads were significantly different between the upper and lower respiratory tract specimens (Table 11). For respiratory tract samples, both upper respiratory tract (nasopharynx + oropharynx swab samples) and lower respiratory tract (sputum samples) viral loads were assayed for their correlation with disease severity. For all upper and lower respiratory tract samples, both *E* and *RdRp* gene targets were subjected to RT-qPCR to check the viral load according to the disease classification and sample collection date (Table 11).

Table 11. Viral RNA copy numbers in upper and lower respiratory-tract specimens

Participants		Upper respiratory tract viral load		Lower respiratory tract viral load	
		E-gene	*RdRp*-gene	*E*-gene	*RdRp*-gene
Asymptomatic	Admission (N = 9)	$6{\cdot}14 \times 10^5$	$7{\cdot}69 \times 10^5$	$1{\cdot}19 \times 10^5$	$9{\cdot}32 \times 10^4$
	Week 1 (N = 7)	$4{\cdot}60 \times 10^4$	$3{\cdot}33 \times 10^3$	$5{\cdot}48 \times 10^4$	$5{\cdot}93 \times 10^4$
	Week 2 (N = 3)	ND	$7{\cdot}02 \times 10^2$	ND	ND
Mild to Moderate	Admission (N = 60)	$9{\cdot}35 \times 10^7$	$1{\cdot}24 \times 10^8$	$2{\cdot}45 \times 10^7$	$3{\cdot}27 \times 10^7$
	Week 1 (N = 55)	$8{\cdot}25 \times 10^6$	$9{\cdot}11 \times 10^6$	$1{\cdot}22 \times 10^7$	$2{\cdot}20 \times 10^7$
	Week 2 (N = 29)	$8{\cdot}77 \times 10^5$	$7{\cdot}18 \times 10^5$	$1{\cdot}15 \times 10^6$	$1{\cdot}44 \times 10^6$
Severe	Admission (N = 8)	$5{\cdot}70 \times 10^6$	$1{\cdot}86 \times 10^7$	$2{\cdot}86 \times 10^6$	$4{\cdot}72 \times 10^6$
	Week 1 (N = 8)	$8{\cdot}45 \times 10^6$	$8{\cdot}78 \times 10^6$	$1{\cdot}93 \times 10^6$	$1{\cdot}34 \times 10^6$
	Week 2 (N = 8)	$3{\cdot}18 \times 10^5$	$3{\cdot}52 \times 10^5$	$2{\cdot}21 \times 10^6$	$3{\cdot}16 \times 10^6$
Critical or Fatal	Admission (N = 18)	$3{\cdot}42 \times 10^8$	$3{\cdot}42 \times 10^8$	$6{\cdot}15 \times 10^7$	$1{\cdot}01 \times 10^8$
	Week 1 (N = 17)	$1{\cdot}61 \times 10^6$	$1{\cdot}61 \times 10^6$	$8{\cdot}35 \times 10^6$	$1{\cdot}16 \times 10^7$
	Week 2 (N = 14)	$4{\cdot}99 \times 10^5$	$7{\cdot}13 \times 10^5$	$2{\cdot}37 \times 10^6$	$2{\cdot}61 \times 10^6$

ND: not detectable; N: number of patients; mean cycle threshold values obtained under standard and viral loads are presented here. Cycle threshold >40 was considered positive according to the instructions of the Kogene Kit and SD Kit

On admission, the viral copy number (6.14×10^5) of the upper respiratory tract (nasopharynx+ oropharynx samples) was considerably higher than that of the lower respiratory tract (sputum) samples (1.19×10^5) (r = 0·47, $P < 0.001$). For asymptomatic patients, the viral RNA copy number ranged from 6 to 7×10^5 on admission and decreased to $4–5 \times 10^4$ in week 1 and gradually disappeared in week 2 after hospitalization. Moreover, in mild to moderate patients, an average of 10×10^7, 10×10^6, and $7–8 \times 10^5$ viral RNA copy numbers were found on admission, week 1, and week 2 samples, respectively. In contrast, high viral RNA loads on respiratory samples of critical or fatal cases were detected in the on admission samples ($3·5–6 \times 10^8$). Similarly, high viral loads were observed in both the week 1 and 2 samples of critical or fatal cases compared with those of the other groups (Table 11).

Furthermore, the correlation between on admission RNAemia and on admission upper respiratory tract samples was r = 0·22 ($P = 0·013$), whereas that with lower respiratory tract samples was r = 0·26 ($P = 0·003$). The correlation of week 1 RNAemia with week 1 upper respiratory tract samples was r = 0·22 ($P = 0·012$), whereas that with week 1 sputum samples was r = 0·32 ($P < 0·001$) (Figure. 8). Similarly, for the critical or fatal cases, the correlation of RNAemia with on admission and week 1 samples of the upper respiratory tract samples was r = 0·53, $P < 0·001$ and r = 0·60, $P < 0·001$, respectively, and that for the lower respiratory tract samples was r = 0·31, $P = 0·047$ and r = 0·43, $P = 0·005$, respectively (Figure.

9). In summary, viral RNAemia was correlated with viral load in respiratory samples.

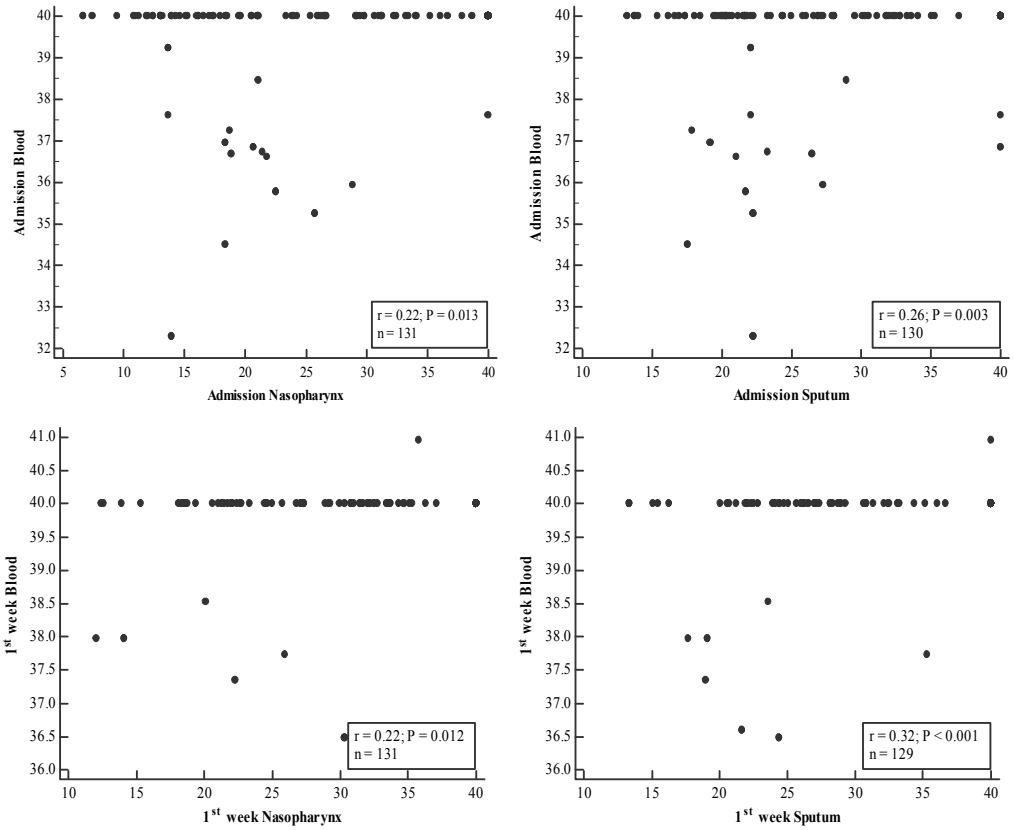


Figure 8: Correlation of blood vs respiratory samples in patients with COVID-19. Correlation of blood vs nasopharynx and sputum of all on admission and week 1 samples. Pearson's correlation coefficient was generated and $P < 0.05$ is considered significant.

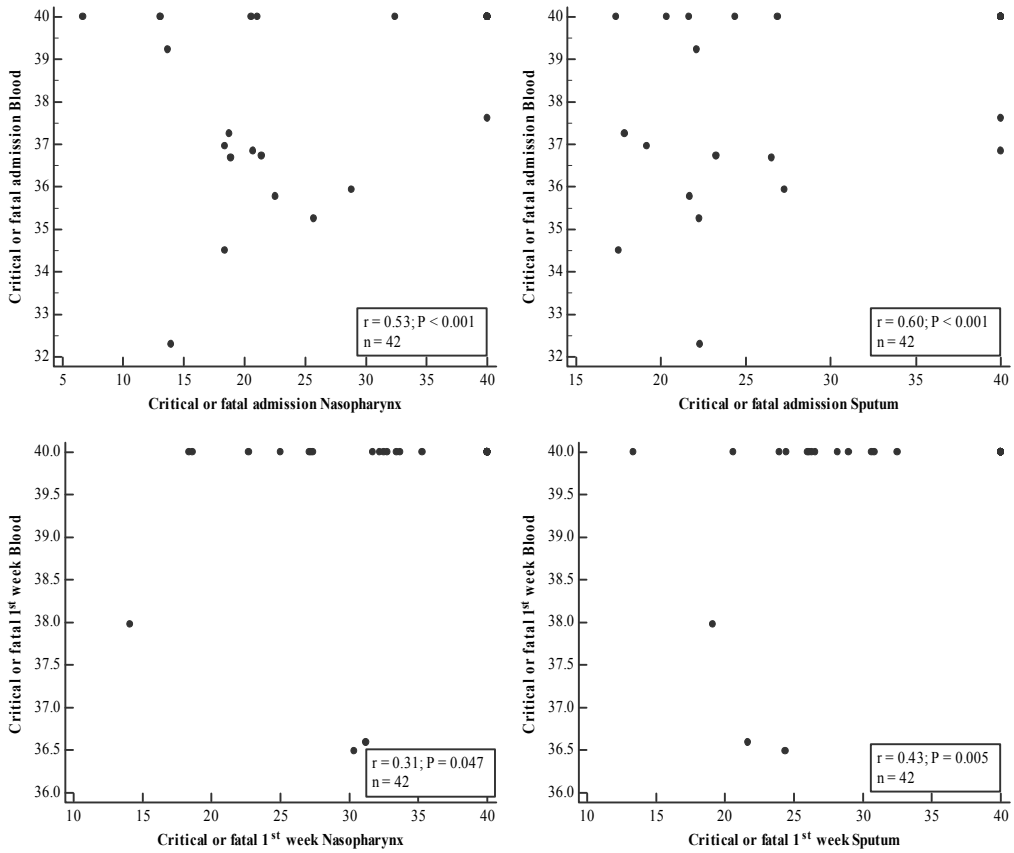


Figure 9: Correlation of blood vs respiratory samples in patients with critical and fatal cases of COVID-19. Correlation of blood vs nasopharynx and sputum of critical or fatal cases. Pearson's correlation coefficient was generated and $P < 0.05$ is considered significant.

4.2.6. Clinical risk factors in association with RNAemia

The clinical correlation between RNAemia and severity was evaluated using baseline risk factors such as age, sex, and other physical and clinical parameters. Univariate logistic regression analysis showed that age, upper respiratory viral copy number, white blood cell count, neutrophil/lymphocyte ratio, and C-reactive protein

were significant ($P < 0.05$); thus, these parameters were considered to be risk factors and were further subjected to multivariate logistic regression analysis. Results of the multiple regression analysis showed that RNAemia and age were predictors of mortality (Table 12).

Table 12. Univariate logistic regression analysis of predictive risk factors for RNAemia

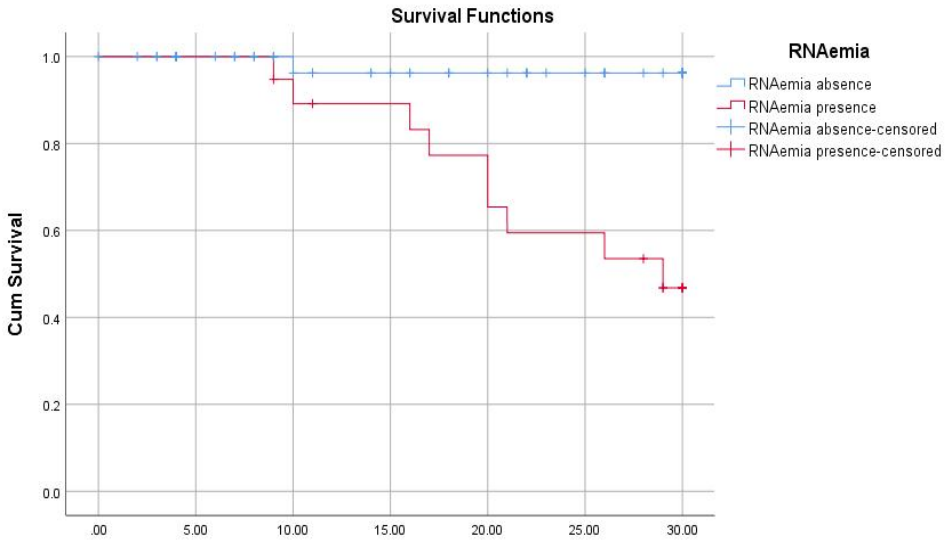
Clinical attributes	Univariate logistic regression analysis		Multiple logistic regression analysis	
	Odds ratio (95% CI)	*P*-value*	Odds ratio (95% CI)	*P*-value
Age	1·201(1·078–1·338)	0·001	1·305(1·061–1·604)	0·012
Male	0·732(0·215–2·492)	0·617		
RNAemia	41·111(7·751–218·044)	<0·001	17·301(1·786–167·551)	0·014
Upper RT viral *E* gene copy number	1(1–1)	0·076		
Upper RT viral *RdRP* gene copy number	1(1–1)	0·065		
Lower RT viral *E* gene copy number	1(1–1)	0·532		
Lower RT viral *RdRP* gene copy number	1(1–1)	0·517		
WBC × 10^9 per L	1·153(0·982–1·354)	0·083		
Neutrophil (%)	1·085(1·026–1·147)	0·004		
Lymphocyte (%)	0·905(0.840–0.975)	0.009		
Neutrophil/Lymphocyte ratio	1.111(1·031–1·197)	0·006	1·108(0·901–1·363)	0·330
C-reactive protein (mg/dL)	1·161(1·060–1·271)	0·001	1·083(0·899–1·305)	1·083
Troponin-I (ng/mL)	126·079(0·177–89663·520)	0·149		
Aspartate aminotransferase (U/L)	1·023(1·004–1·042)	0·018	1·039(0·989–1·090)	0·126
Alanine aminotransferase (U/L)	0·986(0·943–1·031)	0·542		

* $P < 0·05$ is considered significant. CI, confidence interval; CRP, C-reactive protein; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

Disease severity and RNAemia were directly proportional to patient's higher age. Similarly, other clinical features such as C-reactive protein, white blood cell count, neutrophil count, and correlation with upper respiratory tract sample viral loads

directly influenced the presence of viral RNAemia and disease severity.

Hence, our results confirmed the presence of RNA in blood samples during the initial stage of infection, especially in the older population, which might be a marker of disease severity.



RNAemia absence		RNAemia presence		P Value
n	30-day Survival Time (Mean, 95% CI)	n	30-day Survival Time (Mean, 95% CI)	(Log Rank)
76	29.25(28.22-30.27)	19	24.15(20.82-27.47)	<0.001

Figure 10: Kaplan-Meier curve for mortality using patients with RNAemia and non-RNAemia. P-values comparing patients with COVID-19 with evidence of RNAemia to patients without RNAemia were calculated using the Mann-Whitney U test or Fisher's exact test, as appropriate. $P < 0.05$ was considered significant.

The risk factors and mortality rates of patients with and without RNAemia are presented using the Kaplan–Meier curves in Figure 10. Thus, our results predicted a higher mortality rate in patients with RNAemia in correlation with other risk factors.

4.3. SARS-CoV2 Antigenemia as a prognostic markers in COVID 19 patients

4.3.1. Demographic and clinical characteristics of COVID-19 patients in assessment of antigenemia

For the analysis of antigenemia we recruited 119 patients who are clinically confirmed with COVID-19, admitted and treated at Chosun University Hospital, Gwangju, South Korea from February 21, 2020 to October 22, 2021. The median (IQR) age of the patients was 66.0 years, (52.0-79.0) whereas the median age of survival and non-survival patients group age were 62 (48.5-74.3) and 83 (79.0-89.0) respectively. The percentages of men and women patients were 46.2% and 53.8%. Among patients with underlying comorbidities, 38.7% patients had hypertension followed by 25.2% with diabetes mellitus, overall for about 64.7% of patients had some underlying comorbidities. The detailed characteristics are presented in Table 13. On admission, several patients had symptoms such as fever (37%), cough (34.5%), headache (7.6%), chills (16%), sore throat (15.1%), and myalgia (13.4%). In addition, 51.3% of patients underwent supplemental oxygen with 27.7 % with high oxygen flow, whereas 16.8% required mechanical ventilation. Whereas with the treatment scenario, 47% of the patients received antiviral treatment, and 36% patients underwent steroid therapy. Furthermore for about 83% of patients had

antigenemia in their peripheral blood, whereas 27.7% of patients had an elevated antigenemia of which fatal group had the highest percentage of antigenemia elevation (64.7%). For about 23.5% of patients had RNAemia with highest of 76.5% in the fatal group patients as presented in Table 13.

Table 13. Clinical characteristics of COVID-19 patients of antigenemia study

Characteristics	Total (N=119)		Survival group (n=102)		Fatal group (n=17)		*P* value
	n	%	n	%	n	%	
Male gender	55	46.2%	49	48.0%	6	35.3%	0.329
Age, median (IQR)	66.0	52.0-79.0	62.00	48.5-74.3	83.00	79.0-89.0	<0.001
Comorbidities, N (%)	77	64.7%	62	60.8%	15	88.2%	0.028
Cardiovascular disease	16	13.4%	14	13.7%	2	11.8%	1.000
Diabetes mellitus	30	25.2%	22	21.6%	8	47.1%	0.035
Hypertension	46	38.7%	36	35.3%	10	58.8%	0.065
Chronic lung disease	3	2.5%	1	1.0%	2	11.8%	0.053
Cancer	8	6.7%	7	6.9%	1	5.9%	1.000
Chronic kidney disease	2	1.7%	2	2.0%	0	0.0%	1.000
Severity PSI*, Mean ±SD	66.32	24.930	62.44	23.453	92.92	17.863	<0.001
Symptoms							
Fever	44	37.0%	38	37.3%	6	35.3%	0.877
Cough	41	34.5%	35	34.3%	6	35.3%	0.937
Headache	9	7.6%	9	8.8%	0	0.0%	0.355
Chill	19	16.0%	19	18.6%	0	0.0%	0.071
Sore throat	18	15.1%	17	16.7%	1	5.9%	0.464
Myalgia	16	13.4%	16	15.7%	0	0.0%	0.123
Treatments							
Oxygen inhalation	61	51.3%	44	43.1%	17	100.0%	<0.001
High flow oxygen therapy	33	27.7%	17	16.7%	16	94.1%	<0.001
Mechanical ventilation	20	16.8%	7	6.9%	13	76.5%	<0.001
Antiviral	56	47.1%	40	39.2%	16	94.1%	<0.001
Steroids	43	36.1%	28	27.5%	15	88.2%	<0.001

Antigenemia	99	83.2%	83	81.4%	16	94.1%	0.299
No elevation	67	56.3%	63	61.8%	4	23.5%	0.001
Elevation	33	27.7%	22	21.6%	11	64.7%	
Unknown of elevation	19	16.0%	17	16.7%	2	11.8%	
RNAemia	28	23.5%	15	14.7%	13	76.5%	<0.001

Data are expressed as the mean±SD or N (%). The data consisted of all 117 patients who participated in the study. N: number of patients; %: percentage; SD: standard deviation. *missing data total n=17 (survival 13 / fatal 4)

4.3.2. Laboratory characteristics of patients with COVID-19 on admission

The biochemical and laboratory diagnostic characteristics were determined in all patients in both survival and fatal patients. Owing to the results the percentages of white blood cells in survival and fatal cases were 5.8 and 6.4 (10^3 /mL) respectively whereas an elevated WBC were observed in fatal cases. Similar results of elevated biochemical and laboratory findings were observed on other laboratory variables. In contrast, a sharp decrease in lymphocyte count was observed in fatal cases. The detailed biochemical and laboratory variables findings were summarized in Table 14.

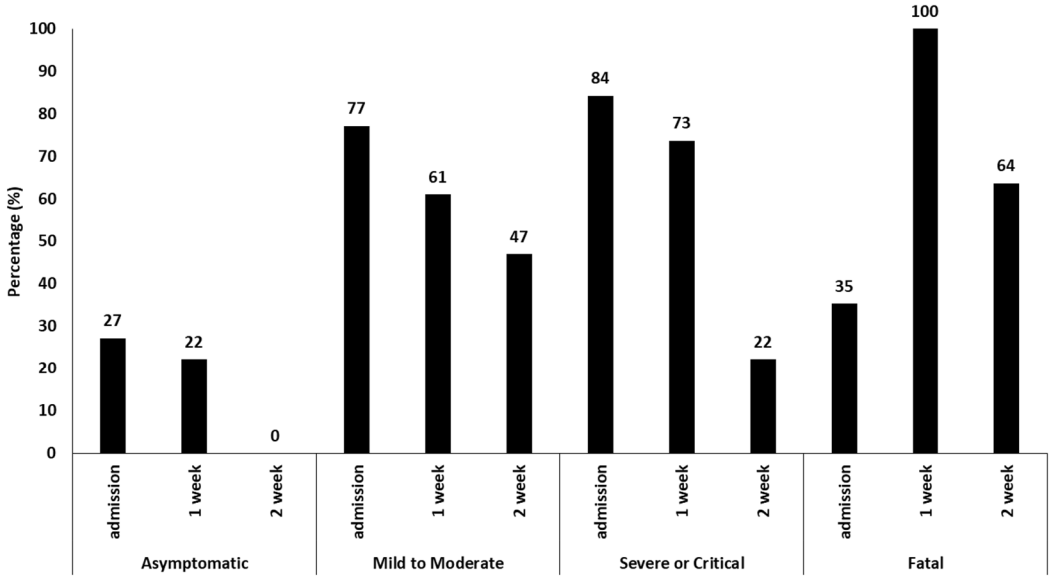
Table 14. Laboratory findings of COVID-19 patients on admission in antigenemia study

Characteristics	Total (N=119)			Survival group (n=102)			Fatal group (n=17)			*P* value
	n	median	IQR	n	median	IQR	n	median	IQR	
WBC (10^3/mL)	118	5.9	4.5-7.2	101	5.8	4.4-7.0	17	6.4	4.9-8.5	0.201
Neutrophils (%)*	118	70.15	±14.22	101	68.08	±13.77	17	82.45	±10.26	<0.001
Lymphocytes (%)*	118	21.32	±11.77	101	22.87	±11.63	17	12.08	±7.79	<0.001
Monocyte (%)*	118	7.37	±3.41	101	7.75	±3.32	17	5.12	±3.15	0.003
Eosinophil (%)	118	0.3	0.0-1.5	101	0.5	0.1-1.8	17	0.0	0.0-0.0	<0.001
Hgb(g/dL)*	117	13.06	±1.69	101	13.24	±1.64	17	12.05	±1.60	0.006
PLT	118	206.0	158.0-244.0	101	212.0	170.5-255.5	17	134.0	122.5-184.0	<0.001
CRP(mg/dL)	105	1.7	0.2-7.1	88	0.8	0.2-6.2	17	9.3	3.6-19.9	<0.001
Troponin-I(ng/mL)	79	0.0	0.0-0.0	64	0.0	0.0-0.0	15	0.0	0.0-0.1	<0.001
AST(U/L)	118	26.4	18.7-44.1	101	24.1	18.1-40.9	17	56.8	30.0-67.3	0.003
ALT(U/L)	118	20.6	12.0-30.5	101	20.7	12.0-30.8	17	17.6	12.5-29.0	0.893
Creatinine(mg/dL)*	118	0.97	±1.24	101	0.96	±1.32	17	1.05	±0.56	0.790
Procalcitonin(ng/mL)	103	0.1	0.0-0.1	86	0.0	0.0-0.1	17	0.2	0.1-0.7	0.001
Fibrinogen(mg/dL)*	82	405.27	±120.06	68	401.84	±117.02	14	421.93	±137.37	0.572
D-dimer	**85**	282.0	153.0-524.0	70	226.0	139.5-474.8	15	478.0	299.0-885.0	0.002
NLR	118	3.6	1.9-6.5	101	3.1	1.8-6.0	17	6.3	4.0-18.1	<0.001
CK-MB(ng/mL)	101	1.4	0.9-2.5	84	1.4	0.8-2.1	17	2.3	1.4-5.2	0.007
Potassium(mEq/L)	115	3.9	3.6-4.2	98	3.9	3.6-4.2	17	4.2	3.8-4.6	0.066

*Described by mean ± SD. N, number of patients; L, liter; WBC, white blood cells; Hb, hemoglobin; CRP, C-reactive protein; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

4.3.3. Evaluation of viral N-protein antigenemia in serum/plasma samples

The patients were categorized to four sub groups namely, asymptomatic, mild to moderate, severe or critical and fatal according to the Sixth Revised Trial Version of the Novel Coronavirus Pneumonia Diagnosis and Treatment Guidance. A total of 364 serum/plasma samples from 119 COVID-19 patients and 81 healthy subjects were assayed for the presence of the viral nucleocapsid protein antigenemia of SARS-CoV-2 using an antigen capture sandwich ELISA. The percentage of the presence of antigenemia on admission and week 1 samples of asymptomatic patients were 27.27% and 22.22% respectively, however none of the samples on week 2 asymptomatic patients detected any antigenemia (Figure 11).

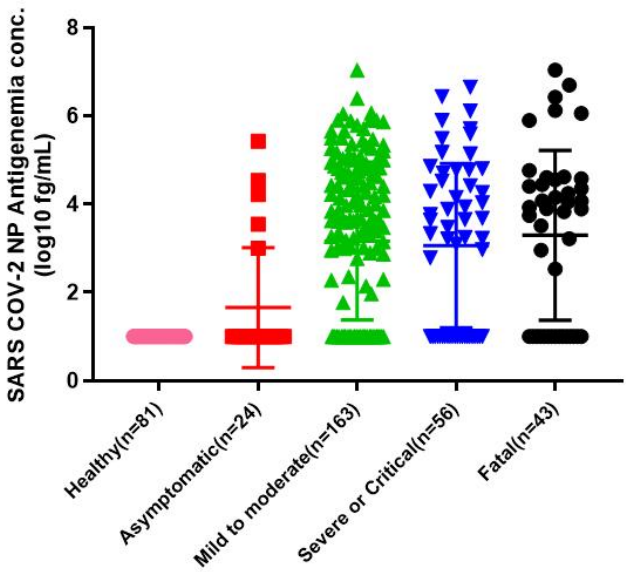


Asymptomatic(n=24)			mild to moderate(n=163)			Severe or Critical(n=56)			Fatal(n=43)		
Admission (n=11)	1st week (n=9)	2nd week (n=4)	Admission (n=70)	1st week (n=59)	2nd week (n=34)	Admission (n=19)	1st week (n=19)	2nd week (n=18)	Admission(n =17)	1st week (n=15)	2nd week (n=11)
27.27	22.22	0.00	77.14	61.02	47.06	84.21	73.68	22.22	35.29	100.00	63.64

Figure 11: Direct percentage and proposition of viral antigenemia in accordance with disease severity. The patient's samples were classified according to the Sixth Revised Trial Version of the Novel Coronavirus Pneumonia Diagnosis and Treatment Guidance, and the data are expressed as N (%).

In the mild-to-moderate category, the detection of antigenemia on admission samples was 77%, in week 1 post hospital admission samples was 61% and the week 2 post hospitalization was 47%. Similarly the proportion of antigenemia in severely ill or critical patients were 84% on admission, 74% on week 1 samples and 22% on week 2 samples. Interestingly, the proportion of antigenemia in fatal patients was low on admission (35%) and the antigenemia peaked to the highest of 100% in the

week 1 samples and declined to 64% in week 2 as shown in Figure 11.



Antigenemia conc. (log10 fg/mL)	Healthy[a] (n=81)	Asymptomatic[b] (n=24)	Mild to moderate[c] (n=163)	Severe or Critical[d] (n=56)	Fatal[e] (n=43)	ANOVA P value	Scheffe's P value
median	1.0(1.0-1.0)	1.0(1.0-1.0)	3.47(1.0-4.72)	3.29(1.0-4.66)	3.89(1.0-4.55)	<0.001	<0.001 a,c / a,d / a,e
mean	1.0(1.0-1.0)	1.66(1.0-1.0)	3.17(1.0-4.72)	3.06(1.0-4.66)	3.29(1.0-4.55)		<0.05 b,c / b,d / b,e

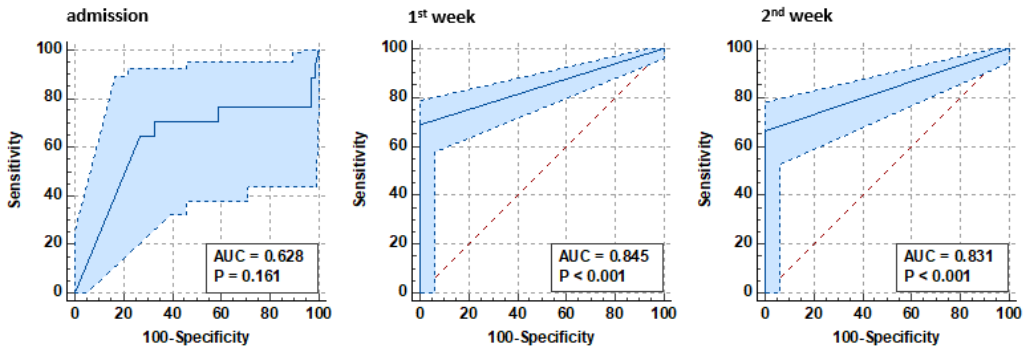
Figure 12: Concentration of antigenemia according to disease severity. The patient's samples were classified according to the Sixth Revised Trial Version of the Novel Coronavirus Pneumonia Diagnosis and Treatment Guidance. The data are expressed in both mean and median. $P < 0.05$ was considered statistically significant

However we attempted to measure the antigenemia concentration according to the classified patients, whereas the median concentration of N-protein antigen stood below 1fg/mL in both healthy and asymptomatic patients. In contrast, for the mild to moderate, and severe and critical cases, the antigenemia levels were higher with a median of 3.47 and 3.29 fg/mL respectively. However in fatal cases the antigenemia

concentration were the highest with a median of 3.89 fg/mL. Therefore we assayed the mean distribution of antigenemia using one-way analysis of variance (ANOVA) followed by posthoc analysis (*Scheffe's*), resulted that antigenemia level is significantly different according to disease severity ($P < 0.001$) as presented in Figure 12.

4.3.4. Investigation of the sensitivity and specificity of antigenemia and upper and lower respiratory track specimens

In order to examine the accuracy of the results we evaluate the sensitivity and specificity using the area under the receiver operating characteristic curve (ROC). The sensitivity and specificity of admission samples antigenemia was 64.71% and 73%. Similarly the sensitivity of 1st week and 2nd week samples antigenemia were 69.05% and 66.29% and the specificity is 100% respectively as shown in Figure 13.



Variables	Admission	1st week (7 days)	2nd week(14 days)
Area under the ROC curve (AUC)	0.628	0.845	0.831
Standard Error	0.0910	0.0207	0.0252
95% Confidence interval	0.533 to 0.715	0.789 to 0.892	0.767 to 0.884
Z statistic	1.403	16.699	13.155
Significance level P (Area=0.5)	0.1606	<0.0001	<0.0001
Younden index J	0.3771	0.6905	0.6629
Associated criterion	≤0.01	>0.01	>0.01
Sensitivity	64.71	69.05	66.29
Specificity	73.00	100.00	100.00

Figure 13. The specificity and sensitivity of antigenemia. a) Determination of specificity and sensitivity on admission samples for antigenemia. b) Determination of specificity and sensitivity on 1st week samples for antigenemia. c) Determination of specificity and sensitivity on 2nd week samples for antigenemia. The data's were presented with ROC curve. $P < 0.05$ was considered statistically.

Mean time the sensitivity of antigenemia was assayed in accordance with time interval from the day of symptom onset to week 2 post symptom onset. Our results clearly demonstrate that the first week of infection possess the highest sensitivity for antigenemia and the sensitivity drastically reduce after 15days of post symptom onset. Meantime RT-qPCR was performed targeting the *E*, and *RdRp* genes using Kogene Kit to assay the sensitivity of upper (nasopharyngeal) and lower (sputum) respiratory tract specimens of all patients with antigenemia according to time interval

form symptom onset to recovery phase. Furthermore while correlating the results of antigenemia with both upper and lower respiratory track viral load, the sensitivity started to decrease in second week. The results were summarized in Table 15.

Table 15. Sensitivity of antigenemia and in upper and lower respiratory-tract specimens at different time point.

Symptom Onset	COVID-19 Antigenemia				Upper respiratory track E gene [Kogene kit]				Lower respiratory track E gene [Kogene kit]			
	Positive	Negative	Total	Sensitivity	Positive	Negative	Total	Sensitivity	Positive	Negative	Total	Sensitivity
-3 – 0	17	6	23	73.9	9	2	11	81.8	9	0	9	100.0
1 – 3	29	4	33	87.9	14	1	15	93.3	8	2	10	80.0
4 – 6	18	9	27	66.7	17	4	21	80.9	9	5	14	64.3
7 – 9	35	13	48	72.9	11	6	17	64.7	13	1	14	92.9
10 – 12	21	9	30	70.0	14	2	16	87.5	6	7	13	46.2
13 – 15	28	13	41	68.3	10	3	13	76.9	8	4	12	66.7
16 –	20	37	57	35.1	16	38	54	29.6	22	31	53	41.5

Samples were segregated as from admission date with 3 days interval until 16+ days; cycle threshold (cutoff) of 35; C_t-35, was used according to Kogene kit manufacturing instruction. For upper respiratory track nasopharyngeal samples and for lower respiratory track sputum samples were used.

4.3.5. Examination of viral RNAemia and antigenemia in serum/plasma samples

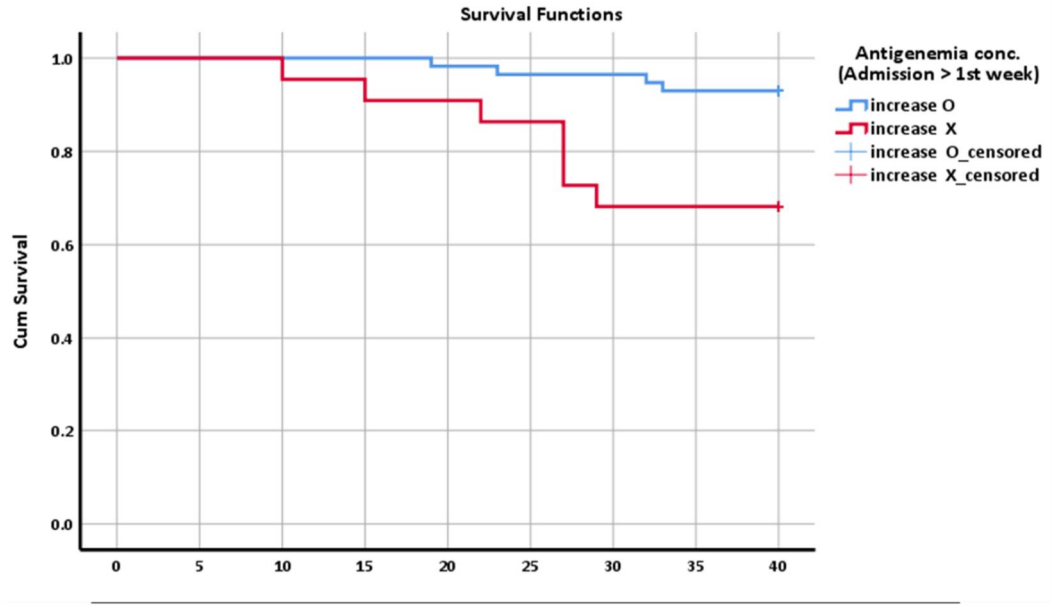
The SARS-CoV-2 viral RNA in blood admission, first-week, and second-week samples were assayed using RT-qPCR targeting *NP* gene. Similarly the antigenemia

presence were also assayed. All samples including, healthy, asymptomatic, mild to moderate, severe or critical and fatal cases were assessed using RT-qPCR to identify the presence of RNAemia and antigen capture ELISA for antigenemia. Viral RNAemia particles were not detected in asymptomatic patients during the entire study period, and none of the samples in second week possess any RNAemia as presented in Table 16. Similarly, the antigenemia and RNAemia concentration in plasma samples of fatal cases were substantially higher in both admission and first-week samples than in other groups of patients. Moreover, a one-way analysis of variance, followed by the Scheffé *post hoc* criterion, for the viral load of RNAemia and antigenemia in different category patients was performed and was statistically significant ($P < 0.001$) for admission and first week samples as mentioned in Table 16.

Table 16. RNAemia and Antigenemia presence in serum/plasm of COVID-19 patients

	Severity	RNAemia			Antigenemia		
		N (%)	ANOVA P value	Scheffe's P value	N (%)	ANOVA P value	Scheffe's P value
Admission	Asymptomatic[a] (N=11)	0 (0%)	<0.001	<0.001a,d/ b,d 0.002b,c 0.027a,c	3 (27.27%)	<0.001	0.006b,d 0.007a,b 0.009a.c 0.011c,d
	Mild to Moderate[b] (N=70)	3 (4.29%)			54 (77.14%)		
	Severe or critical[c] (N=19)	7 (36.84%)			16 (84.21%)		
	Fatal[d] (N=17)	11 (64.71%)			6 (35.29%)		
1 week	Asymptomatic[a] (N=9)	0 (0%)	0.059		2 (22.22%)	0.001	0.047a,c 0.001a,d 0.031b,d
	Mild to Moderate[b] (N=59)	4 (6.78%)			59 (100%)		
	Severe or critical[c] (N=19)	1 (5.26%)			14 (73.68%)		
	Fatal[d] (N=15)	4 (26.67%)			15 (100%)		
2 week	Asymptomatic[a] (N=4)	0 (0%)	NA		0 (0%)	0.039	NA
	Mild to Moderate[b] (N=34)	0 (0%)			16 (47.06%)		
	Severe or critical[c] (N=18)	0 (0%)			4 (22.22%)		
	Fatal[d] (N=11)	0 (0%)			7 (63.64%)		

Data are expressed as mean ± SD or N (%). N: number of patients; NA: not applicable. First sample is on admission, 1st week follow-up sample collected from 5th to 9th day of admission, 2nd week- sample collected from 12th to 16th day of admission ∗ Antigenemia and RNAemia comparisons among multiple subgroups were performed using one-way analysis of variance (ANOVA) followed by Scheffe's *post hoc* test. [a, b, c, d] represents the categories of patients. ∗ $P < 0.05$ indicates statistical significance.



Survival time

Admission > 1st week	Antigenemia increase O		Antigenemia increase X		P Value (Log Rank)
	Total N (event)	40-day Survival Time (Mean, 95% CI)	Total N (event)	40-day Survival Time (Mean, 95% CI)	
	33(11)	34.82(31.89-37.74)	67(4)	39.21(38.38-40.04)	0.005

Figure 14: Kaplan-Meier curve for mortality using antigenemia of patient's samples from admission to 1st week. 40 days survival time was set for all not mortality patients. O represents the increase in antigenemia, X represents the stable or decrease in antigenemia concentration, Event represents the mortality. *P*-values comparing COVID-19 patients with evidence of antigenemia increase were calculated using Mann-Whitney U test, or Fisher's exact test, as appropriate. $P < 0.05$ was considered statistically significant.

The risk factors and mortality rates of patients with antigenemia are presented using the Kaplan–Meier curves in Figure 14. Thus, our results predicted higher mortality rate in patients with increased concentration of antigenemia during the follow-up samples than the admission samples. Hence as reported in Figure 14 our results clearly demonstrate the presence of antigenemia in the first week after

admission in fatal cases were more prone to be a severity marker of mortality in COVID-19.

4.3.6. Assessment of the presence of RNAemia and antigenemia concentration and percentage in accordance with time interval from symptom

In order to further confirm the correlation of antigenemia and RNAemia, we further examined the relation between the antigenemia concentration and RNAemia viral load in accordance with SARS-CoV-2 disease category at various time points. The relation between antigenemia concentration and the viral RNAemia concentration were measured from symptom onset to recovery.

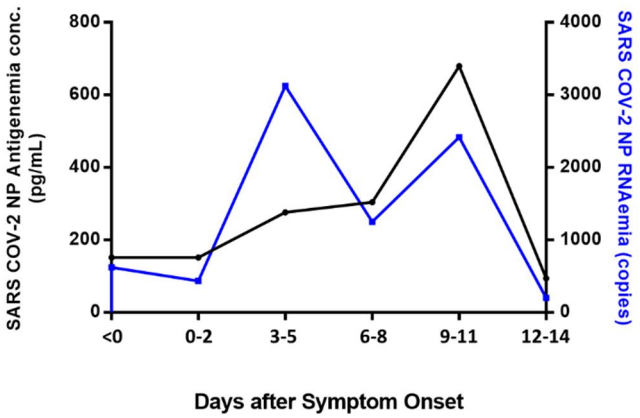
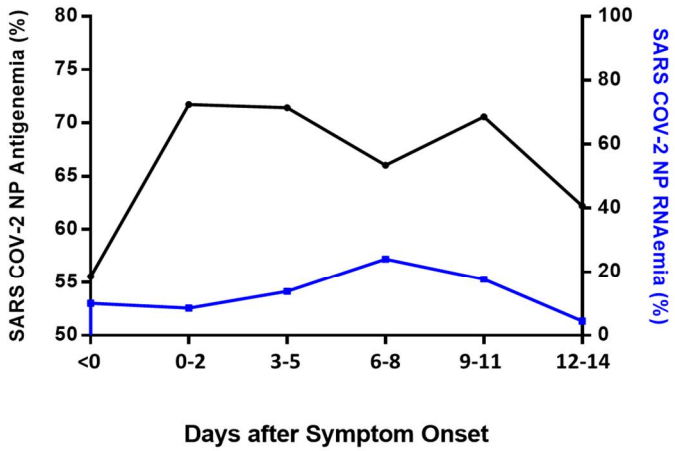


Figure 15: Antigenemia concentration and RNAemia viral copy number with time interval. The concentration of antigenemia and RNAemia viral copy number are presented in mean±SD, the samples were assayed from admission to 2nd week post symptom onset. Antigenemia positive: 0.082pg/mL or more Antigenemia negative: less than 0.082pg/mL. RNAemia positive: showed Ct value with N-gene target RT-qPCR. RNAemia negative: Undetermined in N-gene target RT-qPCR.

As summarized in Figure 15 antigenemia concentration and RNAemia viral copy number well coordinates in accordance with different time interval. Our results shows that, there is an early peak in RNAemia with in 3 to 5 days and decrease, however, for antigenemia, the peaks were observed on about 9 to 11 days after symptom onset. Similarly as shown in Figure 16, we demonstrated the presence of antigenemia and RNAemia in accordance with time interval from the symptom onset. Our results again sows that the percentage of RNAemia drastically reduce on the second week, however for antigenemia after an early peak it gradually decreases.



Days after Symptom onset	-3 – -1	0 - 2	3 - 5	6 - 8	9 - 11	12 - 14
Antigenemia (%)	55.56	71.74	71.43	66.04	70.59	62.22
RNAemia (%)	10.00	8.51	13.79	24.07	17.65	4.44

Figure 16: Antigenemia and RNAemia according to days after symptom onset. The percentage of antigenemia concentration and RNAemia viral copy number are presented as mean±SD. The samples were assayed from admission to 2nd week post symptom onset. Antigenemia positive: 0.082pg/mL or more Antigenemia negative: less than 0.082pg/mL. RNAemia positive: showed Ct value with N-gene target RT-qPCR. RNAemia negative: Undetermined in NP-gene target RT-qPCR.

4.3.7. Evaluation of RNAemia and antigenemia at different time point in accordance with disease severity

To elaborate and further confirm our findings, we further analyzed both antigenemia and RNAemia according to asymptomatic, mild to moderate, severe or critical and fatal in accordance with time interval. The dynamic of viral antigenemia clearly demonstrates that in asymptomatic patients the presence of antigenemia sharply decreased with in the first week and in other categories the concentration of antigenemia were sustained for longer period as presented in Figure 17.

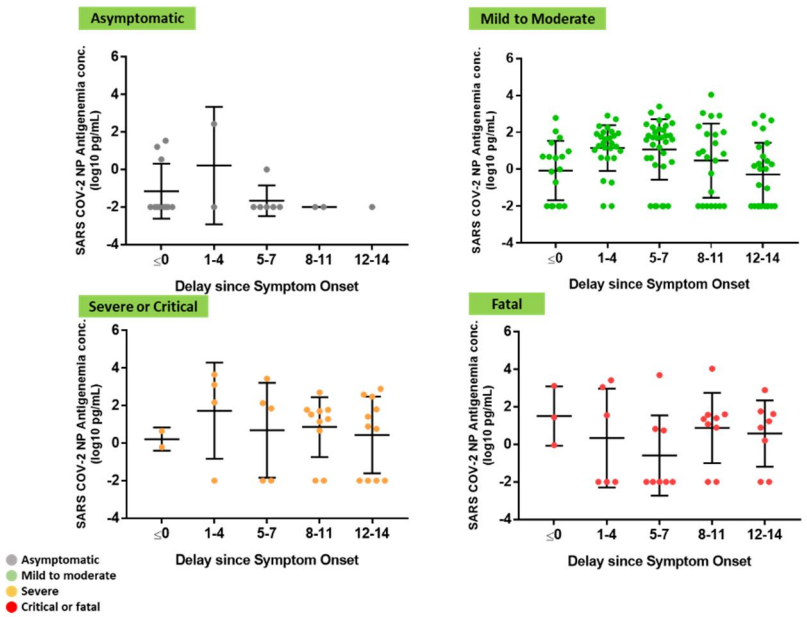


Figure 17: Antigenemia concentration (pg/mL) in accordance with disease severity at different time point. The patient's samples were classified according to the Sixth Revised Trial Version of the Novel Coronavirus Pneumonia Diagnosis and Treatment Guidance. The samples were assayed from admission to 2nd week post symptom onset. The data are expressed in mean±SD.

Furthermore considering the viral RNAemia kinetics, RNAemia was not found in any asymptomatic patients. However in patient with mild to critical cases a decreased pattern of RNAemia were observed in accordance with time interval. On contrary, in fatal cases RNAemia sustained until week 2 as shown in Figure 18. Hence our results clearly portrays that RNAemia and antigenemia were directly correlated with disease severity, specifically in fatal cases where an elevated antigenemia and RNAemia were observed for prolonged period of time.

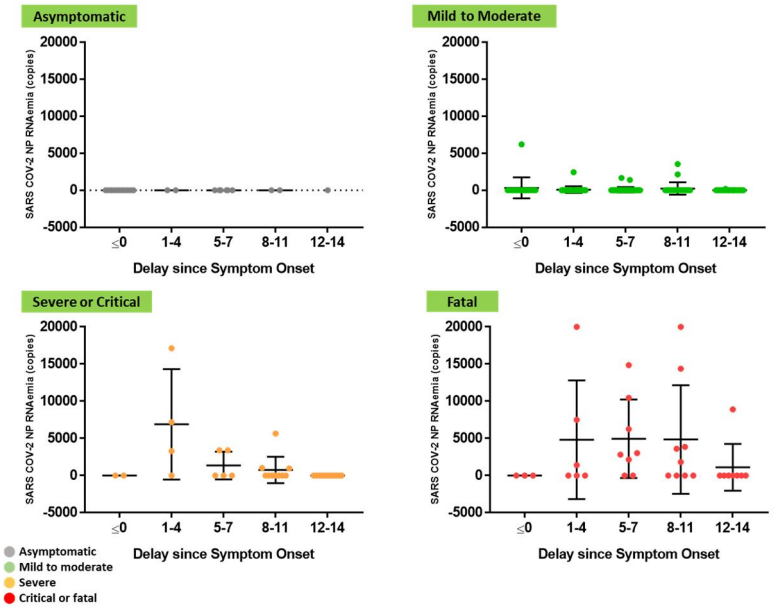


Figure 18: RNAemia copy number in accordance with disease severity at different time point. The patient's samples were classified according to the Sixth Revised Trial Version of the Novel Coronavirus Pneumonia Diagnosis and Treatment Guidance. The samples were assayed from admission to 2nd week post symptom onset. The data are expressed in mean±SD.

4.3.8. Analysis of predictive risk factors for mortality using logistic regression analysis

In addition our results of univariate analysis designates that age, PSI, elevated antigenemia and RNAemia were predictive rick factors of mortality and were statistically significant. Furthermore for multivariate logistic regression analysis, age (adjusted hazard ratio [aHR] 1.11, 95% confidence interval [CI] 1.01–1.21) and RNAemia (aHR 4.78, 95% CI 1.13–20.25) were statistically significant as mortality risk factors.

Table 17. Univariate logistic regression analysis of predictive risk factors for mortality

Variables	Univariate			Multivariate*		
	HR	**95% CI**	***P* value**	**aHR**	**95% CI**	***P* value**
Age	1.14	(1.07 - 1.21)	**< 0.001**	1.11	(1.01 - 1.21)	**0.030**
Comorbidities	3.35	(0.77 - 14.66)	**0.108**	0.23	(0.04 - 1.49)	0.123
PSI	1.05	(1.02 - 1.08)	**0.001**	1.01	(0.97 - 1.06)	0.560
Elevated antigenemia	5.83	(1.86 - 18.31)	**0.003**	2.36	(2.36 - 10.76)	0.267
RNAemia	8.94	(2.91 - 27.51)	**< 0.001**	4.78	(1.13 - 20.25)	**0.034**

*The variables included in the multivariate analysis were age, comorbidities, PSI, Elevated antigenemia, and RNAemia.∗ $P < 0.05$ indicates statistical significance.

4.4. SARS-CoV-2 viral kinetics in accordance with different variants

4.4.1. Patients and samples for variant analysis

A total of 312 specimens including the nasopharynx, oropharynx, sputum and saliva (each 78) were collected from 78 COVID-19 patients. The samples include wild-type and two variants namely delta and omicron. The samples for the assay were collected between 0 to 3rd day of hospital admission of the patients and the samples were analyzed for the presence of viral RNA of SARS-CoV-2 using RT-qPCR.

4.4.2. Examination of SARS-CoV-2 viral load of different variants and specimens using *NP*, *E*, and *RdRp*-gene using RT-qPCR

All samples were subjected to RT-qPCR using NP gene target, however in order to reduce the cost-effectiveness only nasopharyngeal and sputum specimens were analyzed for *E* and *RdRp* target genes. The Ct value was set to be 40 for all the samples. All three groups including wild-type type, delta and omicron variants have a higher viral load on sputum and nasopharyngeal samples using RT-qPCR with NP gene target. Similar RT-qPCR results were observed in nasopharynx and sputum samples using *E* and *RdRp* gene targets. In consistent with our previous results, saliva samples has better sensitivity than oropharynx samples. Furthermore we analyzed

the viral load of different samples (nasopharynx, oropharynx, sputum and saliva), with wild-type type, delta and omicron variants using the nonparametric approach (ANOVA) to identify the difference in the mean distribution of viral load between the variants and samples. The viral load distribution of sputum samples with *E* and *RdRp* gene targets were significant with delta and omicron variants. Similarly the mean distribution was assayed with in the samples in accordance with different variants followed by posthoc analysis (*Scheffe's*). However in our result the distribution of viral load within the delta and omicron variant samples were significant for ANOVA but were not significant with posthoc analysis. Hence our results demonstrated that the viral load of omicron variant with *NP* gene target, did not possess any significant different with wild-type type and delta variants as presented in Table 18.

Table 18. Viral RNA copy numbers and sensitivity and specificity of nasopharynx, sputum, oropharynx and saliva samples.

Participants		Wild[I]			Delta[II]			Omicron[III]			ANOVA P value for viral load		
	Target gene	NP-gene	E-gene	RdRp-gene	NP-gene	E-gene	RdRp-gene	NP-gene	E-gene	RdRp-gene	NP-gene	E-gene	RdRp-gene
Nasopharynx[a]	Viral load	3.75 x 10^6	8.26 x 10^8	8.47 x 10^8	2.27 x 10^7	1.17 x 10^9	1.63 x 10^9	1.20 x 10^7	1.58 x 10^8	1.51 x 10^8	0.537	0.310	0.369
	Sensitivity/ Specificity (AUC)	81.08/100 (0.905)	75.68/100 (0.878)	72.97/100 (0.865)	100/100 (1.000)	95.24/100 (0.976)	95.24/100 (0.976)	100/100 (1.000)	100/100 (1.000)	100/100 (1.000)			
Sputum[b]	Viral load	1.03 x 10^7	3.42 x 10^8	5.56 x 10^8	2.07 x 10^7	2.47 x 10^8	4.88 x 10^8	1.91 x 10^7	1.59 x 10^8	1.38 x 10^8	0.282	0.049	0.010
	Sensitivity/ Specificity (AUC)	94.59/100 (0.973)	64.86/100 (0.824)	62.16/100 (0.865)	95.24/100 (0.976)	90.48/100 (0.952)	90.48/100 (0.952)	100/100 (1.000)	95.00/100 (0.975)	95.00/100 (0.975)			
Oropharynx[c]	Viral load	6.69 x 10^4	NA	NA	3.89 x 10^6	NA	NA	4.48 x 10^4	NA	NA	0.058	NA	NA
	Sensitivity/ Specificity (AUC)	70.27/100 (0.838)	NA	NA	100/100 (1.000)	NA	NA	80/100 (0.900)	NA	NA			
Saliva[d]	Viral load	8.18 x 10^5	NA	NA	2.77 x 10^6	NA	NA	5.69 x 10^5	NA	NA	0.610	NA	NA
	Sensitivity/ Specificity (AUC)	75.68/100 (0.878)	NA	NA	75.0/100 (0.875)	NA	NA	100/100 (1.000)	NA	NA			
P values for viral load	ANOVA	0.288			0.033			0.020					
	Scheffe's	0.43[ac]. 0.45[ad]			0.11[ac]. 0.10[ad]			0.061[ac] . 0.127[ad]					

Samples were collected in between 0–3 days of hospital admission, cycle threshold (cutoff) of 40 (C_t-40) was used for *NP*-gene target. For *E* and *RdRp* gene targets Kogene Kit or SD Kit were utilized according to the manufacturer's instructions. NA: not applicable.

4.4.3. Phylogenetic analysis of partial Spike protein for variant determination

In order to identify the variant classification, the positive amplicons of nested PCR targeting the spike protein were sequenced and further phylogenetically analyzed with Molecular Evolutionary Genetics Analysis (MEGA) software. In addition, the samples collected from the COVID-19 patients before the emergence of variance of concerns (VOC's) such as alpha, beta and gamma variants were considered as wild-type variants in order to differentiate between the delta and omicron type variants. Whereas when the SARS-CoV-2 samples of first wave was send for NGS between 2020-05 to 2021-05 most Korean patients sequencing samples were identified as B.1.497 variant which was present only in Korea. Hence as presented in Figure 19 all the positive amplicon sequences of different variant samples phylogenetically clustered well with the respective reference sequences of wild-type, delta and omicron variants.

In addition the sensitivity/specificity of wild-type, delta and omicron variants samples were assayed using the area under the curve (AUC) by ROC curve. The sensitivity and specificity of the *NP-gene* using Ct 40 for wild-type type sputum samples was 94.59%/100% (AUC- 0.973) and for nasopharyngeal samples was 81.1%/100%, (AUC- 0.905) respectively. For *E-gene* target the sensitivity/specificity of 64.86%/100% (AUC- 0.824) and 75.68%/100% (AUC-0.878) for sputum and nasopharyngeal specimens respectively, and for *RdRp-gene*

target, 62.16%/100%(AUC- 0.865) and 72.97%/100% (AUC- 0.865) for sputum and nasopharyngeal samples respectively. Similarly the sensitivity/specificity of saliva samples using *NP-gene* was 75.68%/100% (AUC- 0.878) for wild-type type, 75.00%/100% (AUC- 0.875) for delta variant and 100%/100% (AUC- 0.100) for omicron variant (Table 18).

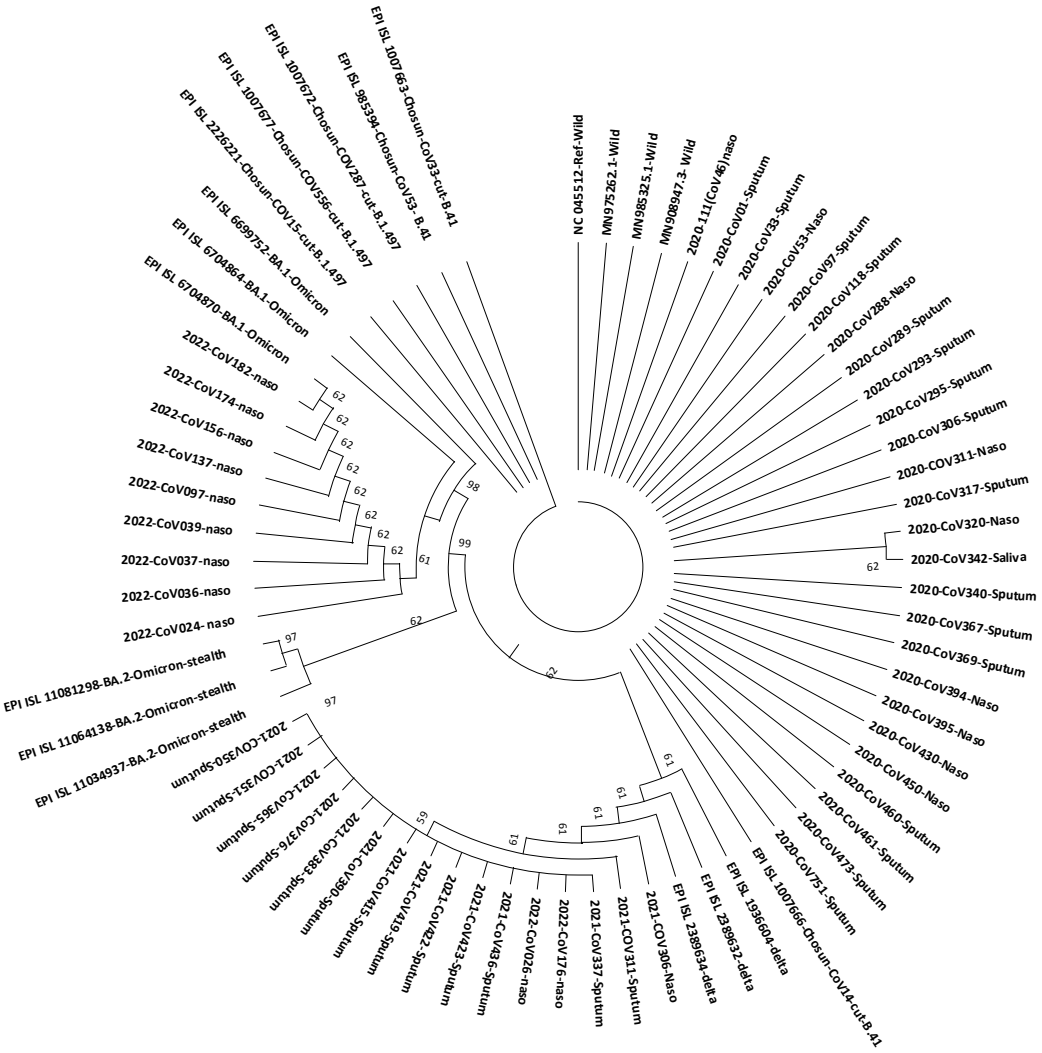


Figure 19: Phylogenetic analysis of Wild, Delta and omicron variant identification. The phylogenetic tree was constructed using the spike gene nester PCR amplicon sequencing, and confirmed the variants with clustering with the respective reference sequences obtained from GISAID database.

4.4.4. Analysis of comparison of specificity and sensitivity of wild-type, delta and omicron variants samples

Furthermore we compared the accuracy according to wild-type, delta and omicron variants samples of *NP* gene target using ROC curve analysis. The comparison of wild-type (AUC- 0.718) vs delta (AUC- 0.987) nasopharynx ($P < 0.001$), wild-type (AUC- 0.718) vs omicron nasopharynx (AUC- 1.000) ($P < 0.001$), wild-type (AUC- 0.558) vs omicron oropharynx (AUC- 0.900) ($P < 0.001$), wild-type (AUC- 0.642) vs delta saliva (AUC- 0.875) ($P < 0.0127$), omicron (AUC- 1.000) vs delta saliva ($P = 0.0118$), wild-type (AUC- 0.652) vs delta sputum (AUC- 1.000) ($P < 0.001$) and wild-type vs omicron sputum (AUC- 1.000) ($P < 0.001$) were clinically significant. In order to evaluate the sensitivity of saliva samples we compared the nasopharynx and sputum samples vs saliva samples of different variants. The sensitivity of nasopharynx and sputum samples of wild-type and delta variants have better sensitivity than saliva samples. However our results also gave that the saliva samples of omicron variants have better sensitivity when compared with the wild-type saliva, nasopharynx and sputum samples ($P < 0.001$) as presented in Figure 20.

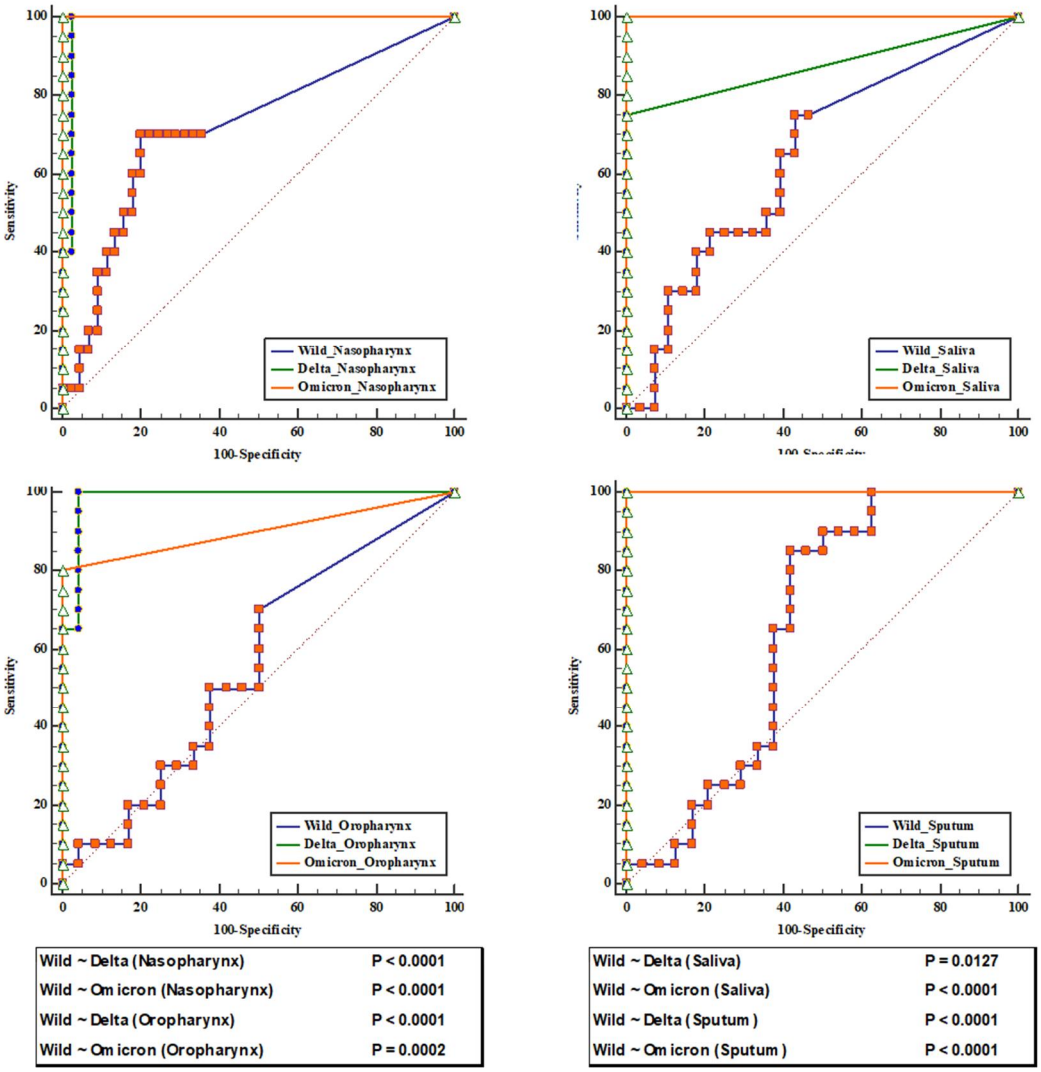


Figure 20: Comparison of the specificity and sensitivity of iNP RT-qPCR among wild-type, delta and omicron variants samples. Comparison of specificity and sensitivity in nasopharynx, saliva, oropharynx and sputum samples with respective of wild-type, delta and omicron variants at c_t cutoff of 40 (c_t-40)

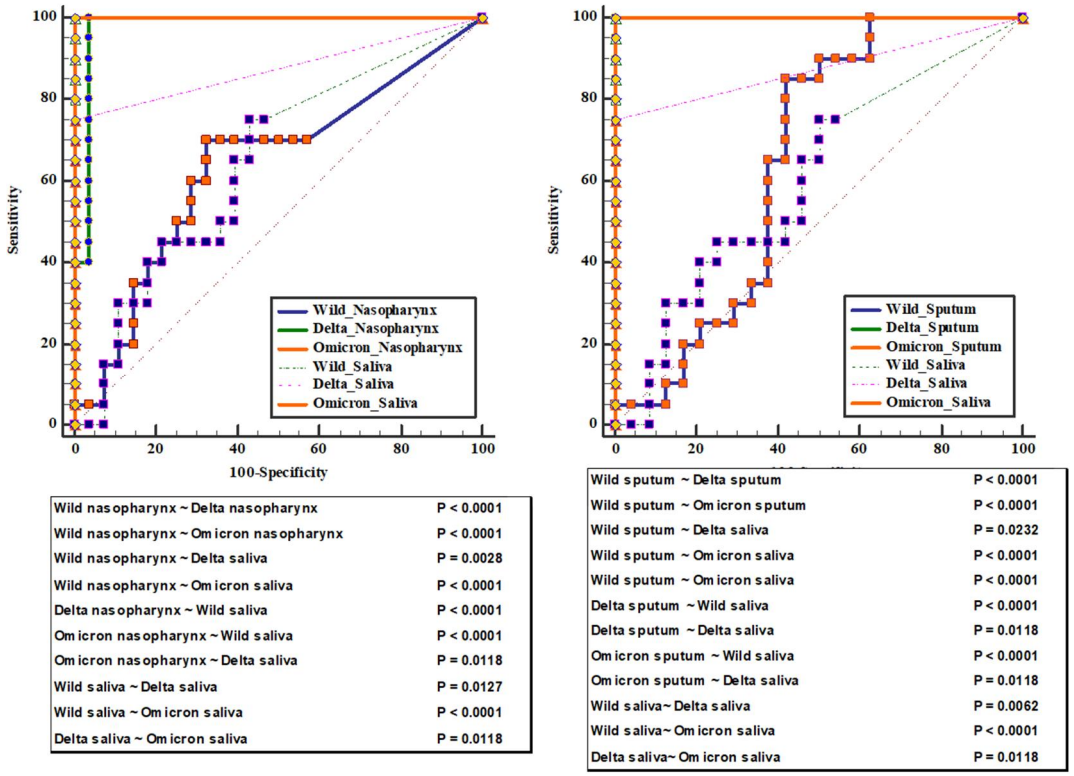


Figure 21: Comparison of the specificity and sensitivity of iNP RT-qPCR between Nasopharynx vs Saliva and Sputum vs Saliva according to variants. Comparison of specificity and sensitivity of wild-type, delta and omicron variants saliva samples verses nasopharynx and sputum samples of wild-type delta and omicron samples.

In addition for further confirmation, we analyzed the comparison of different variant samples in respective with wild-type vs delta, wild-type vs omicron and delta vs omicron as presented in Figure 21. Our results shows that the omicron saliva has better sensitivity compared with other wild-type or delta variant saliva samples ($P < 0.001$). The viral load of saliva samples of wild-type, delta and omicron variants were 8.18 x 10^5, 2.77 x 10^6 and 5.69 x 10^5 respectively, with no

significant difference in viral load as presented in Table 18 ($P = 0.61$).

4.4.5. Assessment of viral RNA copy numbers according to vaccinated and non-vaccinated patients

In addition, we attempted to evaluate the viral copy number variation of SARS-CoV-2 in vaccinated and non-vaccinated patients. None of the wild-type infected patients were vaccinated since vaccine was not supplied in Korea. The viral load of saliva samples using *NP* target gene in vaccinated and non-vaccinated patients with delta variant were 1.57×10^6 and 3.03×10^6 ($P = 0.43$) as presented in Table 19. Furthermore the viral load using *NP* gene target in saliva, sputum and oropharyngeal samples of vaccinated (6.33×10^5, 2.33×10^7 and 5.72×10^4) patients and non-vaccinated (4.49×10^5, 1.31×10^7, and 2.97×10^4) patients with omicron variant did not possess any statistical significances. Even though most of the patients with omicron variant received vaccination, there is no significant difference of viral load according to vaccination status, so further study is necessary to confirm the vaccination effects.

Table 19. Viral RNA copy numbers according to vaccinated and non-vaccinated patients.

Variants	Target gene	Nasopharynx viral load		ANOVA P value	Sputum viral load		ANOVA P value	Oropharynx viral load		ANOVA P value	Saliva viral load		ANOVA P value
		Vaccinated (N)	Non-vaccinated (N)		Vaccinated (N)	Non-vaccinated (N)		Vaccinated (N)	Non-vaccinated (N)		Vaccinated (N)	Non-vaccinated (N)	
Wild	**NP-gene**	NA	3.75×10^6 (30)		NA	1.03×10^7 (35)		NA	6.69×10^4 (27)		NA	8.18×10^5 (28)	
	E-gene	NA	8.26×10^8 (30)		NA	3.42×10^8 (35)		NA	NA		NA	NA	
	RdRp-gene	NA	8.47×10^8 (30)		NA	5.56×10^8 (35)		NA	NA		NA	NA	
Delta	**NP-gene**	2.96×10^7 (4)	2.24×10^7 (17)	0.569	1.68×10^7 (4)	2.15×10^7 (17)	0.424	4.97×10^6 (4)	3.68×10^6 (17)	0.632	1.57×10^6 (4)	3.03×10^6 (17)	0.432
	E-gene	7.85×10^7 (4)	2.19×10^9 (17)	0.332	8.91×10^8 (4)	1.78×10^8 (17)	0.103	NA	NA		NA	NA	
	RdRp-gene	1.44×10^8 (4)	2.89×10^9 (17)	0.296	2.46×10^9 (4)	3.40×10^8 (17)	0.041	NA	NA		NA	NA	
Omicron	**NP-gene**	1.07×10^7 (13)	1.48×10^7 (7)	0.572	2.33×10^7 (13)	1.31×10^7 (7)	0.720	5.72×10^4 (13)	2.97×10^4 (7)	0.461	6.33×10^5 (13)	4.49×10^5 (7)	0.120
	E-gene	2.45×10^8 (13)	7.01×10^7 (7)	0.532	2.22×10^8 (13)	7.42×10^7 (7)	0.159	NA	NA		NA	NA	
	RdRp-gene	8.73×10^7 (13)	4.85×10^8 (7)	0.529	7.19×10^7 (13)	4.59×10^8 (7)	0.419	NA	NA		NA	NA	

V. DISCUSSION

Several studies had reported the diagnostic accuracy of SARS-CoV-2 in confirmed COVID-19 patients with serological and culture-based assays [94, 95, 96]. However, the major differences in analytical specificity and sensitivity among the samples of different human tissues/biological liquids and among different time points after the onset of SARS-CoV-2 have not been addressed. Here I analyzed the results of most available primer sets which revealed that the primer sets need to be reassessed regarding their specificity and sensitivity because they can yield false negative and false positive results and therefore leads to an incorrect diagnosis. The infection of SARS-CoV-2 is widespread in family clusters, on food premises, at workplaces, and religious gatherings where the presence of high viral load in a patients who are relatively mild or asymptomatic at the initial stage of infection [97, 98].

Present day diagnostic methods are based on RT-qPCR or deep-sequencing technologies that requires the replication of a viral RNA to ensure that a sufficient amount of the virus is collected for diagnosis [99]. Under the present scenario, viral RNA detection by RT-qPCR is regarded as one of the principal diagnostic methods for COVID-19 [100]. Nevertheless, the reliability of RT-qPCR has been debated due to false negative and false positive results [101]. In some cases, positive results are confirmed after full recovery or in the absence of infection; for some patients, the COVID-19 diagnosis has been

falsely ruled out based on consecutive negative results of RT-qPCR analysis of respiratory-tract samples [102]. In other cases, the patients were suspected to be SARS-CoV-2 positive according to their clinical presentation and the history of exposure to the disease, but their oropharyngeal and nasopharyngeal swabs repeatedly tested negative with RT-qPCR; eventually, broncho-alveolar lavage fluid was found to be SARS-CoV-2–positive using RT-qPCR on the 8th day [103].

A few studies have assessed self-collected saliva and other samples for diagnosing COVID-19; however, to date, the selection of clinical samples for accurate diagnosis has not been specified accurately [104, 105]. In the present study, 31 saliva samples tested positive, with a sensitivity of 79.1% in the first week of symptom onset and a drastic decline of sensitivity to 37.5% at 2 weeks post symptom onset. None of the saliva samples collected after 3 weeks tested positive throughout our study. Our data are consistent with the previously published results, where high viral load was reported in severe cases and persisted for a long time in clinical samples, whereas, in mild cases of COVID-19, high viral load is detectable at the initial stage and can disappear at the later stage [106].

One study suggests that the rate of SARS-CoV-2 positivity in assays of sputum samples is significantly higher than that for throat swabs, and sputum samples may be of greater value for diagnosing COVID-19 [107]. Moreover,

in our study, nasopharyngeal samples showed a sharp decline in RT-qPCR sensitivity within 1 week, whereas sensitivity in sputum samples was consistent for ~2 weeks and gradually declined thereafter. The Infectious Diseases Society of America (IDSA) suggests the use of nasopharyngeal, mid-turbinate, or nasal samples rather than oropharyngeal or saliva samples for COVID-19 diagnosis [108]. However, the suitability of Upper-respiratory-tract samples remains uncertain [109]. CDC recommends the use of oropharyngeal specimens collected by a health care professionals, leading to certain discrepancy between the guidelines set forth by CDC and IDSA [110]. In our study, I proved that the oropharyngeal samples demonstrated the lowest sensitivity for the detection of SARS-CoV-2. A recent study provides evidence that in the detection of SARS-CoV-2, saliva samples may yield higher sensitivity than nasopharyngeal samples [111]. Moreover, the present study revealed that iNP RT-qPCR analysis of self-collected saliva samples has higher sensitivity as compared with oropharyngeal samples during the first week after symptom onset.

SARS-CoV-2 RNA in blood, known as RNAemia, and its effect on disease severity and fatal clinical outcomes, is not fully understood. The case fatality rate for COVID-19 varies by age, with 0·3 deaths per 1000 cases among the young (aged between 5 to 17 years old) to 304·9 deaths per 1000 cases in patients aged > 85 years [112]. In a previous study, a qualitative viral detection

assay in plasma showed correlation with disease severity [113]. Another study, which was performed by adapting the published real-time RT-qPCR assays targeting *E* gene showed that RNAemia was present in approximately one-third of patient's samples. [114].

A few studies have reported RNAemia and disease severity in COVID-19 patients with other clinical aspects, including risk factors such as age, smoking, and comorbidities; however, they failed to reflect the disease progression and severity correlated with viral RNAemia. [115, 116]. I explored the correlation between RNA viral loads along with various demographic, laboratory, biochemical, physical, and clinical parameters using 95 clinically confirmed patients with COVID-19. Moreover, we evaluated the viral load from the detectable SARS-CoV-2 RNA in the upper respiratory tract swab and lower respiratory tract samples along with SARS-CoV-2 RNA in plasma in association with other clinical parameters of COVID-19 characteristics and severity.

The results are consistent with previously published data, where viral RNAemia was higher in critically ill patients than in mild to severe patients and very rare in outpatients [117]. The percentage of RNAemia in critical or fatal cases was the highest (67%), followed by severe (13%) and mild to moderate (2%) cases on the admission samples of the patients. Similarly, RNAemia was detected in the week 1 samples (critical or fatal [18%], severe

[13%], and mild to moderate [5%]). However, RNAemia was not detected in any of the samples collected from week 2 after symptom onset. Furthermore, none of the asymptomatic patients exhibited RNAemia throughout the study period. The viral load of blood samples was lower than that of the upper and lower respiratory tract samples, and considerably higher viral copy numbers were detected in critical and fatal case on admission samples than the admission samples of severe and mild to moderate cases. Using logistic regression analysis, I showed that RNAemia was a risk factor for disease severity in COVID-19 patients. Simultaneously, this study provides evidence that the presence of RNAemia is associated with a higher mortality rate than that in non-RNAemia cases.

SARS-CoV-2 nucleocapsid protein antigenemia in blood and their relationship on disease severity and fatal outcomes, is not fully understood. Similarly the correlation and kinetic comparison of antigenemia and viral RNAemia were not studied well. However the correlation of antigenemia and its clinical relevance with RNAemia were also not well documented. In a previous study on human cytomegalovirus (HCMV) infections, monitoring of pp65 antigenemia was compared with the results of quantitative PCR of the nucleic acids [118]. In an asymptomatic patient with SARS-CoV1 in early 2004 where antigenemia and seroconversion was well documented [119]. In a case study reported previously where there was persistent antigenemia and

RNAemia was documented for a prolonged period since the post-symptom onset [120]. Several studies have reported the disease severity in COVID-19 patients with many clinical aspects, including other risk factors such as age, smoking, and various other comorbidities; however, they failed to reflect the disease progression and the severity correlated with viral antigenemia and RNAemia [121, 122, 123, 124].

Hence I explored the risk of antigenemia and RNA viral load along with various demographic, laboratory, biochemical, physical, and clinical parameters with clinically confirmed patients with COVID-19. Furthermore we evaluated the viral RNA in upper and lower respiratory track samples in association with antigenemia. As reported in previous studies SARS-CoV-2 viral *NP*-antigenemia and RNAemia independently associated with fatal clinical outcome had been reported but the study was limited to ICU patient's alone [125]. Antigenemia, RNAemia and various DNA PCR are widely used for monitoring SARS-CoV2 viral infections around the globe. Owing to various clinical and severity markers of SARS-CoV2, it is important to study the sensitivity and comparison between the markers and its clinical usefulness in diagnosis and to predict the disease severity.

In this study I comprehensively analyzed the kinetics of viral *NP*-protein antigenemia in accordance with the disease severity along with clinical classification and in accordance with time dependent manner with COVID-19

patients. In the prospective of viral antigenemia kinetics, our results provide a solid evidence that the antigenemia peaked in the first week and start descending in the second week. Furthermore I demonstrated the potential relation between the elevated antigenemia concentration and RNAemia in the mortality outcome using univariate logistic regression analysis for predictive risk factors. I further confirmed that antigenemia concentration and RNAemia viral load is elevated in severe to critical patients, however both antigenemia and RNAemia is having the highest concentration in fatal cases. The data presented in the study are relevant to clinical pathological implication and connected well with the predictive risk factor of mortality outcome.

Marais *et.al* reported that omicron variants had a high viral shedding in saliva compared with nasopharyngeal samples which results in improved diagnostic performance on saliva samples and suggested that the upper respiratory tract tissue possibly may have altered tissue tropism [126]. In our previous study we analyzed the sensitivity and specificity of various clinical specimens, where the sensitivity of sputum sample was the highest followed by nasopharyngeal, saliva then oropharyngeal specimens, in which all the specimens were wild-type [127]. Furthermore, in another previous study, we also assayed the viral kinetics of SARS-CoV-2 infection and compared the viral kinetics with symptoms, treatment and disease severity, where the viral load is significantly higher in the patients who received steroid therapy [128].

In the present study I demonstrated the viral load of different SARS-CoV-2 variants and analyzed the difference in viral load of vaccinated and non-vaccinated patients with COVID-19. Furthermore we analyzed the viral load of wild-type and delta and omicron variants with respective to various clinical specimens including nasopharynx, oropharynx, sputum and saliva. Another study in United Kingdom reported that higher viral load of omicron may cause the increased transmission of viral particles by aerosolization [129]. However our results showed a contrast results where there is no significant difference in viral load with omicron, delta or wild-type variants. Our results were consistent with the previous published results where higher viral load doesn't support the rapid spread of omicron variants, where the viral load of omicron is lower than delta variants and suggested that for its fast spread might be bypassing the immunity generated by previous infection or vaccination [130]. Omicron's infectiousness may not be linked with the higher viral load measured in the upper or lower respiratory track specimens because viral RNA have a shorter clearance phase and lower viral load than delta infections, besides, the study also suggested that lower viral load may also be associated with vaccination [131]. In another recent study, they confirmed that the omicron variant infection is not triggered or enhanced by transmembrane serine protease 2 (TMPRSS2) rather the enhancement is mainly mediated by endocytic pathways. Hence the different infective pathway of omicron variant

may have its own clinical implication and infective ability [132].

A recent computational docking study addressed that in omicron variant spike protein RBD regions 468–473 having a disorder transition and may be significantly improve the stability and binding to ACE2 may be a reason for higher infectivity [133]. In another study the spike and ACE2 complex reveals a new salt bridges and hydrogen bonds with mutated residues of R493, S496 and R498, and neutralization assays exhibit increased antibody evasion together with strong interactions with ACE2 interface may likely contribute the rapid spread of the omicron variant [134]. Furthermore an another study reported that the correlation of infectious viral load in vaccinated individuals with delta variant infected patients is relatively low when compared with unvaccinated patients where a 4.5 fold higher infectious viral load is observed, furthermore suggesting omicron variant did not show elevated infectious viral load, hence suggesting other mechanism may contribute to rapid infection with omicron variants [135]. In our study also there is no significant difference in viral load with vaccinated and non-vaccinated patients. In a recent report suggests that saliva samples of omicron variant is superior when compared with nasopharyngeal and oropharyngeal samples [136]. Similar results were observed in a community study where they suggest that self-collected saliva may have good sensitivity and reliable for wide scale screening for COVID19 [137]. Our results is also consistent with the study where the omicron saliva

also possess better sensitivity along with nasopharyngeal and sputum samples. In addition, there is no significant difference in viral load with vaccinated and non-vaccinated patients infected with omicron variants.

VI. CONCLUSIONS

In conclusion, this study demonstrates the analytical and diagnostic specificity and sensitivity of various RT-qPCR primers used in clinical practice for detection of SARS-CoV-2 in COVID-19 patients. Moreover we defined that, sputum samples yielded the highest sensitivity for RT-qPCRs, followed by nasopharyngeal, saliva, and oropharyngeal samples. Furthermore, we report with evidence that the CDC and WHO primers need considerable improvement for more accurate detection of SARS-CoV-2.

My finding also discussed the quantitative determination of blood RNAemia with clinical significance in COVID-19 patients. Using logistic regression analysis, we showed that RNAemia was a risk factor for disease severity. Simultaneously, this study provides evidence that the presence of RNAemia is associated with higher mortality rate than that of non-RNAemia cases.

I comprehensively analyzed the kinetics of viral *NP*-protein antigenemia and other clinical factors in accordance with the disease severity along with clinical classification with time dependent manner in COVID-19 patients. Furthermore we demonstrated the potential relation and correlation between the antigenemia concentration and RNAemia viral load in blood in the mortality outcome. Our results predicated that fatal cases possess the highest concentration of antigenemia in the first week than admission, while other group of patients possess a decreased tendency in antigenemia concentration

in follow-up samples. In addition I also demonstrated the predictive rick factors using univariate and multivariate logistic regression analysis of mortality, which include age, comorbidities, PSI, antigenemia and RNAemia.

Furthermore, our study confirms that the saliva sample of omicron variant has higher sensitivity compared with delta and wild-type saliva samples even though there is no significant difference on viral copy number in wild-type, delta or omicron variants irrespective of vaccinated or non-vaccinated status of the patients. In addition our assay comparisons with different variants gave that saliva samples of omicron have better sensitivity compared with wild-type or delta variant nasopharynx and sputum specimens.

VII. REFERENCES

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