Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.
May viral load detected in saliva in the early stages of infection be a prognostic indicator in COVID-19 patients?

Sukru Aydin a, *, Isilay Gokce Benk b, Aysegul Altintop Geckil c

a Malatya Training and Research Hospital, Department of Otorhinolaryngology, Malatya, Turkey
b Malatya Training and Research Hospital, Department of Clinical Microbiology, Malatya, Turkey
c Malatya Training and Research Hospital, Department of Pulmonary Disease, Malatya, Turkey

ARTICLE INFO

Keywords:
SARS-CoV-2
Viral load
Saliva
Prognosis

ABSTRACT

Objective: This study aimed to investigate the prognostic value of viral load detected in the saliva of COVID-19 patients in the early stage of infection.

Study design: Oro-nasopharyngeal swab and saliva samples were collected from all patients simultaneously in the early stage of COVID-19. Viral loads were determined by extracting viral RNAs from saliva samples of patients whose ONP swabs were positive for SARS-CoV-2 by RT-qPCR. The demographic information, comorbidities, cycle threshold values, and one-month clinical courses were recorded and compared.

Results: The patients’ clinical course was evaluated for one month; 56 % of patients had mild disease, 26.4 % had moderate disease, 9.6 % had severe disease, and 8% had a critical/mortal disease. The average cycle threshold values of SARS-CoV-2 in saliva and ONP samples were measured as 22.28 and 24.19, respectively. Cycle threshold value of saliva was found to be significant in predicting disease severity (Eta coefficient 0.979). A statistically significant relationship was found between the disease’s severity and the mean of ONP samples’ Ct-values (p < 0.05). Gender, age, body mass index, and co-morbidities were compared with the severity of the disease; no statistically significant difference was found.

Conclusion: Viral load detected in saliva in the early period of COVID-19 infection may have a prognostic value in showing the disease’s course in patients over 45-year-old. Saliva is an easily obtainable, reliable material for COVID-19 screening.

1. Introduction

Coronavirus Disease-2019 (COVID-19) spread worldwide shortly after it was first identified in Wuhan-China in November 2019, and it has become a world-threatening disease caused by a newly identified coronavirus (nCoV-2019) (WHO, 2020a). It was described as a new pandemic by the World Health Organization (WHO) on March 11, 2020, and the virus was named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (WHO, 2020b). According to WHO data, the pandemic has reached 76,745,892 total cases and 1,694,300 total deaths in the whole world by December 20, 2020 (WHO, 2020c).

COVID-19 enforces the limits of nations’ health systems by causing severe infection in about 13.8 % and mortality in about 4% of the patients (Sun et al., 2020). In studies conducted to predict the course of COVID-19 patients, older age and having co-morbidities (chronic obstructive pulmonary disease (COPD), cardiovascular disease (CVD), arterial hypertension (HT)) have been shown as poor prognostic factors; however, all of the data remains insufficient in predicting the clinical course of patients (Sun et al., 2020; Wang et al., 2020). It is crucial to determine the factors that will predict which patients will develop viral pneumonia, in which patients viral pneumonia will progress to acute respiratory distress syndrome (ARDS), and which patients will have a mortal course.

Saliva contains particularly high amounts of SARS-CoV-2 in the early period of infection; however, the high amount of virus’s origin and prognostic significance in this period has not been adequately clarified. High viral load in the saliva can be explained by the fact that SARS-CoV-2 infects target cells in the oro-nasopharyngeal tissues in the early period and interferes with the salivary secretion by replication in the target cells (Xu et al., 2020b). Although the disease’s pathobiology is not fully understood, it seems that in the early period of infection, oropharyngeal target cells are the ductal epithelium of minor salivary glands rich in

* Corresponding author.
E-mail addresses: dr.sukruaydin@gmail.com (S. Aydin), gokce-benk@hotmail.com (I.G. Benk), aysegul.altintop@gmail.com (A.A. Geckil).

https://doi.org/10.1016/j.jviromet.2021.114198
Received 17 January 2021; Received in revised form 16 April 2021; Accepted 22 May 2021
Available online 24 May 2021
0166-0934/© 2021 Elsevier B.V. All rights reserved.
angiotensin-converting enzyme 2 (ACE2) receptor, and ACE2 receptor-rich epithelial cells of the oral mucosa (Liu et al., 2011; Xu et al., 2020a, b). SARS-CoV-2 enters target cells with its spike protein that has a strong affinity to human ACE2, and transmembrane protease serine 2 (TMPRSS2) is needed to entry and viral spread of SARS-CoV-2 through interaction with the ACE2 receptor (Hoffmann et al., 2020; Zhang et al., 2020). After reaching the lungs, the virus can cause morbidity and mortality by affecting type-II alveolar epithelium in the lung that causing viral pneumonia. The occurrence of viral pneumonia could be explained by the fact that the type-II alveolar epithelium lining the alveoli is rich from ACE2 receptors, which are the targets of SARS-CoV-2; by the way, the lung loses an essential protective mechanism after the blockage of the ACE2 receptor pathway (Imai et al., 2005). Viral spread can also reach endothelial cells, kidney tissue cells, intestinal cells and increase morbidity and mortality (Crackower et al., 2002; Danilczyk et al., 2006; Gu et al., 2005; Hamming et al., 2004; Zhao et al., 2020).

In this study –by establishing the hypothesis that the patients with a more severe clinical course should have a higher viral load in their saliva in the early period of COVID-19—we aimed to define patients who will have a poor clinical course.

2. Materials and methods

2.1. Patients and sample collection

Patients who applied to Malatya Training and Research Hospital with suspected COVID-19 infection were included in the study. The study was organized between October 1–30, 2020 and oronasopharyngeal (ONP) samples and saliva samples were collected from all patients simultaneously.

Informed consent forms were obtained from all of the patients who would participate in the study. Only patients older than 45-year-old and whose symptomatic period did not exceed three days between sampling were included.

ONP samples were collected from 300 patients that eligible for the study, as described in the Republic of Turkey Ministry of Health COVID-19 Guideline (Republic of Turkey Ministry of Health, 2020). The samples were transported to the laboratory in a sterile transport tube containing viral nucleic acid extraction buffer (vNAT). ONP samples were stored at room temperature (approximately 25 °C) and delivered to the laboratory on the same day. While saliva samples were collected, patients were warned not to cough and produce sputum, collect only the saliva in the mouth, and then slowly drop it into a sterile container two or three times without bubbles (approximately 500 μL). Patients who did not correctly give saliva samples were excluded from the study. Samples were stored at -20 degrees until the viral load of saliva samples was investigated.

2.2. Viral RNA amplification from ONP samples

ONP samples were examined in Malatya Training and Research Hospital PCR Laboratory. Viral ribonucleic acid (RNA) amplification from ONP samples was performed according to the manufacturer’s instructions using the SARS-CoV-2 Double Gene RT-qPCR (Bio-Speedy-USHAŞ, Ankara, Turkey) kit. Since the Biospeedy RT-qPCR kit is validated with vNAT buffer that provides viral nucleic acid extraction, no further extraction procedure was required. Samples with a cycle threshold (Ct) value below 38 (equivalent to approximately 5,610 virus copies / mL) after amplification were considered positive for SARS-CoV-2. Ct-values of patients were recorded (Ct-value/ONP). Of the 300 patients included in the study, quantitative reverse transcription-polymerase chain reaction (RT-qPCR) results of ONP samples of 128 patients were reported as positive and salivary material of 128 patients were used for the study.

2.3. Preparation of saliva samples

Phosphate-Buffer Saline (PBS) was added to the collected saliva samples in 1 or 2 folds, and the viscosity of the saliva was reduced to provide easy pipetting. The prepared suspension was cold centrifuged at 2000 g for 5 min. Four hundred μL of samples were taken from the supernatant portion for RT-qPCR.

2.4. Extraction and detection of viral RNA from saliva

Viral RNA extraction from prepared saliva samples was performed according to the manufacturer’s instructions using the QIAsymphony RNA (Qiagen, Hilden, Germany) kit. After isolation of viral RNA, amplification of ORF1 ab and N gene fragments of SARS CoV-2 virus was performed with the SARS-CoV-2 Double Gene RT-qPCR (Bio-Speedy-USHAŞ, Ankara, Turkey) kit. Ct-values below 38 were considered positive, and the Ct-values were recorded numerically as a quantitative indicator of viral load (Ct-value/saliva). Viral RNA isolation and amplification were successfully performed in saliva samples of 125 patients, and three patients had Ct-values above 38. The patients with a Ct-value above 38 in saliva were followed up, but were excluded from statistical analyses regarding clinical course.

2.5. Data

The study was conducted with the Malatya Clinical Research Ethics Committee’s permission with the protocol numbered 2020/167 and in compliance with the Helsinki declaration. All patients included in the study were informed, and voluntary consent forms were obtained.

The patients’ age, gender, co-morbidities (diabetes (DM), HT, CVD (Arrhythmia, heart failure, coronary artery disease), Asthma, COPD, malignancy, immunodeficiency), body mass indexes (BMI) were recorded. The clinical course of the patients was recorded by telephone and hospital registry system.

Whether the patients experienced shortness of breath, hospitalizations, oxygen requirements, intensive care admissions, and deaths were recorded.

To group the patients’ clinical course, the Republic of Turkey Ministry of Health COVID-19 Guideline and the study that Sun et al. reported were considered (Republic of Turkey Ministry of Health, 2020; Sun et al., 2020). According to these data, we grouped patients: who had the disease at home and did not experience shortness of breath were evaluated as ‘mild disease,’ patients who experienced shortness of breath for a while during the disease but did not require hospitalization and oxygen were assessed as ‘moderate disease,’ patients who hospitalized or required oxygen; but do not need intensive care were evaluated as ‘severe disease,’ and patients who were admitted to intensive care and/or intubated and/or had a mortal course were grouped as ‘critical/mortal disease.’

2.6. Statistical analysis

Data were given with median (min-max), mean (standard deviation), and number (percentage). The normality of variables was evaluated by the Kolmogorov-Smirnov test. Pearson chi-square test, Yatesin corrected chi-square test, Fisher’s exact chi-square test, Kruskal Wallis test, and One-Way Analysis of Variance test were used where appropriate. Tukey and Tamhane T2 tests were utilized for one-way analysis of variance in multiple comparisons. Correlation between Ct-values and disease severity was analyzed with the Eta coefficient. A p-value of <0.05 was considered a statistically significant difference. IBM SPSS Statistics 17.0 (SPSS Inc., Chicago, IL, USA) program was used for analysis.

3. Results

The average age of the patients included in the study was 62.1
The patients’ average Ct-value/saliva was measured as 22.28 (min: 7.90, max: 33.74, SD: 5). The patients’ average Ct-value/ONP was measured as 24.19 (min: 10.22, max:33.98, SD: 0.43). The clinical course of the patients was evaluated as the result of a one-month follow-up. Seventy of the patients had a disease at home, and dyspnea complaints did not develop during the follow-up period (mild disease), 33 patients complained of shortness of breath or dyspnea; but hospitalization or oxygen requirement did not occur (moderate disease), 12 patients were hospitalized and/or needed oxygen support (severe disease), ten patients required intensive care and showed a fatal course (critical/mortal disease). The clinical course of the patients with a Ct-value over 38 was followed up, the patients had the disease at home and dyspnea complaints did not develop.

The mean of Ct-value/saliva was: 25.3 (SD: 0.41) in the mild disease group, 19.85 (CI: 18.7–21.0) in the moderate disease group, 16.75 (CI:14.4–19.0) in the severe disease group, and 15.48 (CI:11.9–19.0) in the critical/mortal disease group. The mean of Ct-value/ONP was: 26.00 (CI:25.10–26.89) in the mild disease group, 22.95 (CI: 21.22–24.68) in the moderate disease group, 20.12 (CI:17.47–22.77) in the severe disease group, and 20.59 (CI:16.16–25.02) in the critical/mortal group. There is a moderately significant positive correlation between Ct-value/ONP and Ct-value/saliva (r = 0.402, p < 0.0001). The Ct-value/saliva was found to be significant in predicting disease severity (Eta coefficient: 0.979). A statistically significant relationship was found in the ANOVA test between the disease’s severity and the Ct-value/saliva (p < 0.05, ANOVA’s F value: 45.055). Post-Hoc tests were performed with Tukey and Tamhane T2; it was observed that there was no statistically significant difference between the Ct-value/saliva means of severe disease and critical/mortal disease groups (p > 0.05); however, when all other paired groups were compared, it was observed that the Ct-value/saliva means were significantly different from each other (p < 0.05). A statistically significant relationship was found between the disease’s severity and the Ct-value/ONP (p < 0.05). When the gender, age, BMI, and co-morbidities of the patients were compared with the severity of the disease, no statistically significant difference was found (p > 0.05) (Table 1). However, it was a remarkable finding that 8 of the patients whose clinical course was critical/mortal were male.

### Table 1
Relationship of demographic data and comorbidities between groups showing the clinical course of COVID-19.

| Gender | Severity of COVID-19 | P value* (Pearson Chi-square tests) |
|--------|-----------------------|------------------------------------|
|        | All patients n (%)    | Mild disease n (%) | Moderate disease n (%) | Severe disease n (%) | Critical/mortal disease n (%) |
| Male   | 61 (48.8 %)           | 33 (47.1 %)         | 14 (42.4 %)            | 6 (50 %)            | 8 (80 %)                        | 0.211 |
| Female | 64 (51.2 %)           | 37 (52.9 %)         | 19 (57.6 %)            | 6 (50 %)            | 2 (20 %)                        | 0.107** |
| DM‡    | no 87 (69.6 %)        | 53 (75.7 %)         | 23 (69.7 %)            | 7 (58.3 %)          | 4 (40 %)                        | 0.735 |
|        | yes 38 (30.4 %)       | 17 (24.3 %)         | 10 (30.3 %)            | 5 (41.7 %)          | 6 (60 %)                        | 0.36** |
| Hypertension | no 71 (56.8 %) | 42 (60 %)         | 16 (48.5 %)            | 7 (58.3 %)          | 6 (60 %)                        | 0.022** |
|        | yes 54 (43.2 %)       | 28 (40 %)           | 17 (51.5 %)            | 5 (41.7 %)          | 4 (40 %)                        | 0.564** |
| Cardiovascular disease | no 101 (80.8 %) | 57 (81.4 %)         | 30 (90.9 %)            | 9 (75 %)            | 5 (50 %)                        | 0.675** |
|        | yes 24 (19.2 %)       | 13 (18.6 %)         | 3 (9.1 %)              | 3 (25 %)            | 5 (50 %)                        | 0.606** |
| Asthma | no 112 (89.6 %)       | 67 (95.7 %)         | 25 (75.8 %)            | 11 (91.7 %)         | 9 (90 %)                        | |
|        | yes 13 (10.4 %)       | 3 (4.3 %)           | 8 (24.2 %)             | 1 (8.3 %)           | 1 (10 %)                        | |
| COPD‡  | no 120 (96 %)         | 68 (97.1 %)         | 31 (93.9 %)            | 12 (100 %)          | 9 (90 %)                        | |
|        | yes 5 (4 %)           | 2 (2.9 %)           | 2 (6.1 %)              | 0 (0 %)             | 1 (10 %)                        | |
| Malignancy | no 120 (96 %) | 66 (94.3 %)         | 32 (97 %)              | 12 (100 %)          | 10 (100 %)                      | |
|        | yes 5 (4 %)           | 4 (5.7 %)           | 1 (3 %)                | 0 (0 %)             | 0 (0 %)                         | |
| Immune deficiency | no 120 (96 %) | 68 (97.1 %)         | 32 (97 %)              | 11 (91.7 %)         | 9 (90 %)                        | |
|        | yes 5 (4 %)           | 2 (2.9 %)           | 1 (3 %)                | 1 (8.3 %)           | 1 (10 %)                        | |

n¹: count of patients, DM²: diabetes, COPD³: chronic obstructive pulmonary disease; *: a p value is significant when it is lower than 0.05 level, **: More than 20 % of cells in these subtables have expected cell counts less than 5, Chi-square results may be invalid.
relationship between Ct-value and clinical course and prognosis of patients in their study but showed a significant statistical inverse correlation between inflammatory hematological markers C-reactive protein and LDH and Ct-value (Azzi et al., 2020).

There are studies in the literature showing that the viral load in the upper respiratory tract peaked in the first days of onset of symptoms (He et al., 2020). Nagura-Ikeda et al. showed that the viral load decreased significantly in saliva samples taken after the 10th day compared to samples taken in the first nine days of symptoms and reported that the early saliva sample would be more valuable. The same study reported that the viral load in saliva could not be detected in some cases, and it may be due to the late collection of samples (Nagura-Ikeda et al., 2020). Also, Williams et al. reported gradually decreasing salivary Ct-values during days from the onset of the complaints (Williams et al., 2020). Based on this information, it seems essential to collect saliva samples at the beginning of the complaints to show SARS-CoV-2 and determine the viral load amount more accurately.

Liu et al. determined that the laryngopharyngeal minor salivary gland epithelium was the target cell in their study to determine early target cells for SARS-CoV virus in Chinese rhesus macaques. The same study showed that the target cells were infected two days after viral inoculation (Liu et al., 2011). Studies have shown that SARS-CoV and SARS-CoV-2 spike protein show a high homology degree and similar binding properties to the ACE2 receptor (Li et al., 2005; Xu et al., 2020c). In another study, Xu et al. claimed that the oral mucosal epithelium is rich in ACE2 receptors and that early target cells for SARS-CoV-2 may be the oral mucosal epithelium (Xu et al., 2020a). Based on these data, it can be declared for SARS-CoV-2 that the minor salivary gland ductal epithelium and oral mucosal epithelium rich in ACE2 receptor are early target cells, and this fact can explain the high viral load in saliva in the early period.

Identifying the prognostic factors for COVID-19 in the early stage can be life-saving for patients. Advanced age and co-morbidities (especially CVD, HT, COPD) have been shown in studies as poor prognostic factors (Sun et al., 2020). In our study, all patients were over 45-year-old, and no significant difference was found between the clinical course of COVID-19 and age and co-morbidities (p > 0.05). A minimum 45-year-old limiting for our study was based on the fact that the median ages of hospitalized patients varied between 47 and 73 in most of the studies (Docherty et al., 2020; Guan et al., 2020; Richardson et al., 2020). Thus, most of our patients were in the age range at risk of hospitalization. The lack of a statistically significant relationship between advanced age and co-morbidities in this study may be due to the low number of patients included in the research and the absence of patients under 45-year-old in the study.

Detecting SARS-CoV-2 by RT-qPCR from ONP swab or sputum is the gold standard diagnostic method for diagnosing COVID-19 infection; however, this diagnostic test’s sensitivity may be quite low (Sethuraman et al., 2020). In ONP swab samples, the detection sensitivity of SARS-CoV-2 by RT-qPCR was found as 33 % in screening tests performed in persons with contact with SARS-CoV-2, 62 % in screening performed during the symptomatic period, and 80 % in scans performed three days after the first symptom (He et al., 2020; Kucirka et al., 2020; Sethuraman et al., 2020). When the sensitivity of detection of SARS-CoV-2 with RT-qPCR of ONP swab samples and saliva samples were compared, a consistency of 69.2–100 % was reported (Azzi et al., 2020; Becker et al., 2020; Kojima et al., 2020; McCormick-Baw et al., 2020; Pasomsub et al., 2020; To, 2020; To et al., 2020; Wyllie et al., 2020). In our study, SARS-CoV-2 was shown in the saliva samples of 97.6 % of the patients whose ONP swab samples were positive for SARS-CoV-2. Our research and similar previous studies show that the saliva sample is a usable material for SARS-CoV-2 screening and detection.

In this study, a moderate positive correlation was found between ct-value/saliva and ct-value/ONP. The ct-value/saliva was significantly lower than the ct-value/ONP. This suggests that saliva may be a better material to detect viral load than ONP samples, albeit, both seem to be statistically significant in predicting the disease’s course.

There are some limiting factors in our study. Since patients under 45-year-old were not included in our study, the prognostic effect of viral load in the patient group younger than 45 years was not investigated. Since some of the patients survived the disease at home, this group’s clinical course was recorded with patient statements, which may have caused subjective and biased results.

In conclusion, saliva sample seems to be an easily obtainable and reliable material for screening and diagnosing SARS-CoV-2 infection. Viral load detected in saliva in the early symptomatic period of infection may have a prognostic value in showing the course of the disease in patients over 45-year-old, and should be supported by studies with a larger number of patients.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Ethics approval

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Malatya Clinical Research Ethics Committee (protocol number 2020/167).

Consent to participate

Informed consent was obtained from all individual participants included in the study.

Consent for publication

Patients signed informed consent regarding publishing their data.

Author statement

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by [Sukru Aydin], [Isilay Gokce Benk] and [Aysegul Altintop Geckil]. The first draft of the manuscript was written by [Sukru Aydin] and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.
Availability of data and material

The datasets generated during and analyzed during the current study are available from the corresponding author on reasonable request.

We thank to Barış Otuğ, Professor Doctor from Inonu University, Department of Clinical Microbiology, for his help in designing this study.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

Arizi, L., Carcano, G., Gianfagna, F., Gromi, P., Dalla Gasperina, D., Genoni, A., Faasano, M., Sessa, F., Tettamanti, L., Carinci, F., 2020. Saliva is a reliable tool to detect SARS-CoV-2. J. Infect.

Becker, D., Sandoval, E., Amin, A., De Hoff, P., Leonetti, N., Lim, Y.W., Elliott, C., Lau, E.H., Wu, P., Deng, X., Wang, J., Hao, X., Lau, Y.C., Wong, J.Y., Guan, Y., Guan, W.J., Ni, Z.Y., Hu, Y., Liang, W.H., Ou, C.Q., He, J.X., Liu, L., Shan, H., Lei, C.L., Kojima, N., Turner, F., Slepnev, V., Bacelar, A., Deming, L., Kodeboyina, S., Klausner, J.

Azzi, L., Carcano, G., Gianfagna, F., Grossi, P., Dalla Gasperina, D., Genoni, A., Faasano, M., Sessa, F., Tettamanti, L., Carinci, F., 2020. Saliva is a reliable tool to detect SARS-CoV-2. J. Infect.

Docherty, A.B., Harrison, E.M., Green, C.A., Hardwick, H.E., Pius, R., Norman, L., Becker, D., Sandoval, E., Amin, A., De Hoff, P., Leonetti, N., Lim, Y.W., Elliott, C., He, X., Lau, E.H., Wu, P., Deng, X., Wang, J., Hao, X., Lau, Y.C., Wong, J.Y., Guan, Y., Guan, W.J., Ni, Z.Y., Hu, Y., Liang, W.H., Ou, C.Q., He, J.X., Liu, L., Shan, H., Lei, C.L., Kojima, N., Turner, F., Slepnev, V., Bacelar, A., Deming, L., Kodeboyina, S., Klausner, J.

Hamming, I., Timens, W., Bulthuis, M.L., Lely, A.T., Navis, G., van Goor, H., 2004. Tissue invasion by SARS-CoV-2: an observational cohort study. Lancet Infect. Dis. 20, 565–574.

To, K.K., Wu, T.C., Chan, J.C., Yeung, W.-S., Chik, T.S.-H., Choi, C.Y.-C., Kandamby, D.H., 2020. Persistent characteristics, comparisons and outcomes among 5700 patients hospitalized with COVID-19 in the New York City area. JAMA.

Sethuraman, N., Jeremiah, S.S., Ryo, A., 2020. Interpreting diagnostic tests for SARS-CoV-2. JAMA.

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. For the availability of data and material, see the corresponding author on reasonable request.

The datasets generated during and analyzed during the current study are available from the corresponding author on reasonable request.

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The availability of data and material is provided by the corresponding author on reasonable request.

The availability of data and material is provided by the corresponding author on reasonable request.

The availability of data and material is provided by the corresponding author on reasonable request.