Dynamics of SAR-CoV2 Viral Load Decline and Antibody Responses in COVID-19 Patients

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Abstract

This study describes changes in the viral RNA, viable virus shedding and host immune response in COVID-19 patients. Samples showed a large variation of viral load which declined rapidly in the first week and gradually afterward. Most cases with persistent positive of viral RNA (approximate 80%) did not indicate shedding of viable virus. Some cases with undetectable specific antibody (9 from 26 cases) were viral culture positive. These indicate that delaying of immune response may result in persistent shedding of viable virus. High viral load and negative neutralizing antibody may be used as markers to identify such individuals.

Introduction

Many studies showed that viral RNA have been detected in respiratory tract of COVID-19 patients for several weeks [11, 14, 15]. The RT-PCR specific to SAR-CoV2 RNA is used as the standard method of high sensitivity to confirm COVID-19 patient diagnosis, and is able to detect remaining viral RNA with no infectious particles. Present discharge plan following criteria of 2 consecutive negative results of RT-PCR leads to delayed patient discharge and burdens the investigation services, the limited available facilities and increases costs. Although one study showed that viable viruses were not found in prolonged RT-PCR positive patients [11].

For various types of viruses, prolonged viral infection patients cannot get rid of viruses leading to chronic illness. Studies revealed some COVID-19 patients have persistence illness for weeks to months despite the betterment of COVID-19 ailment. This “long COVID” symptoms can vary and manifest in many body systems, for example; nervous system (confusion, insomnia), respiratory system (dyspnea, sore throat), gastrointestinal system (diarrhea), other such as fatigue. These symptoms were considered as complication after viral clearance [1, 10]. However, there is no evidence shows certain viral replication within the body to be the cause of these symptoms.

Neutralizing antibody is supposed to be important mechanism for viral clearance. Though, no evidence indicates that extended shedding of viable viruses is related to inability to or less neutralizing antibody production. In contrary, reports showed relation between neutralizing antibody level and severity of disease; patients with severe conditions have higher neutralizing antibody than those with mild and no symptom. These may be due to, severe condition patients have high viral load, inducing more inflammation and hence, high antibody responses [2, 4, 6]. Other report showed the relation of slow neutralizing antibody response to severity of symptoms, which suggested that not only neutralizing antibody level but also duration of neutralizing antibody production was important for viral clearance and severity of symptoms [3].

Persistence of viral RNA in respiratory samples of COVID-19 patients and asymptotically infected individuals is a common and important problem in the outbreak control. The main question is whether the persistence of viral RNA reflects viable virus shedding. And, if there is viable virus shedding, there is an interesting scientific question of how the virus survives in the presence of immune responses, which should be developed soon after the infection. Possible explanations include a failure of developing immune responses or immune escape by viral mutations. To answer these questions, this project performed viral isolation in sequential respiratory samples and antibody detection in serum samples of mild-symptom or asymptomatic patients, who showed viral RNA positive by RT-PCR 1 week after diagnosis.

Materials And Methods

This research project has been approved from Siriraj Institutional Review Board (SIRB), Faculty of Medicine, Siriraj Hospital, Mahidol University. Nasopharyngeal swab and venous blood samples were collected every 3-7 days from 92 cases of RT-PCR confirmed COVID-19 patients who were admitted in Nakhon Pathom hospital, Siriraj hospital and Golden Jubilee Medical center during December 2020 to January 2021 and their RT-PCR results still positive more than a week.

SARS-CoV-2 isolation was performed in biosafety level 3 laboratory and research project has been biosafety approved by Mahidol University Institutional Biosafety Committee (MU-IBC). Vero E6 cell line (ATCC CRL-1586) was used for viral isolation. Cell was cultured in Dulbecco's Modified Eagles's Medium (DMEM; Gibco) with 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin (Pen/Strep; Gibco) at 37°C+5%CO₂. Sample was adsorbed to 18-hour cultured Vero E6 cell for 1 hour at 37°C+5%CO₂,

then DMEM with 2%FBS was added and incubated at 37°C+5%CO₂ for 2-7 days. The infected cells were observed by cytopathic effect (CPE) and SARS-CoV-2 antigen detection using immunofluorescence assay. Supernatant of viral culture was collected and titrated by TCID₅₀ (50% tissue infectious dose) in Vero E6 cell.

SARS-CoV2 antigen in infected cells were detected by immunofluorescence assay. Two to seven-day SARS-CoV-2 infected Vero E6 cells were fixed by 80% acetone and were washed by 0.03% Triton X (Sigma) in PBS, then were blocked by 3% Bovine serum albumin (BSA; Sigma) in PBS. SARS-CoV/SARS-CoV-2 nucleocapsid rabbit monoclonal antibody (Sino Biological) was added and incubated at 37°C for 1 hour. After incubation, washing was performed, then Alexa Fluor 488 conjugated goat anti rabbit IgG (Invitrogen) and Hoechst 33342 (Invitrogen) were added and incubated for 1 hour at 37°C. After washing, cells were fixed by 1% paraformaldehyde, then were observed under inverted fluorescent microscope.

Viral RNA was extracted from viral transport media (VTM) using Liferiver® RNA isolation kit according to manufactural protocol. The real time RT-PCR for envelope (E) gene of SAR-CoV-2 was performed by laboratory of faculty of medical technology, Mahidol University.

SARS-CoV-2 specific antibody in serum was detected by in-house ELISA. The SARS-CoV-2 RBD protein (Sino Biological) coated MaxiSorp Nunc-immuno 96-well plate was blocked by 5% BSA in PBS with 0.1% Tween-20. Two thousand-fold diluted serum was added and incubated at 37°C for 1 hour. After incubation, plate was washed and HRP-conjugated goat anti human IgG antibody (Thermo) was added and incubated for 1 hour at 37°C, then plate was washed. KPL TMB microwell peroxidase substrate (Seracare) was added and reaction was stopped by 1M H₂SO₄. The absorbance at 450/630 nm was measured using microplate reader. The average with one standard deviation of absorbance values from eighty-eight SAR-CoV-2 negative serum (collected in 2010) was used as cut off value.

Micro neutralization assay (micro NT) was performed to detect neutralizing antibody. Two-fold serial dilution from 1:10 to 1:1280 of serum samples in DMEM with 2%FBS and 1%Pen/Strep, were incubated with equal volume of 100TCID SAR-CoV-2 at 37°C+5%CO₂ for 1 hour. The mixture was added to 18-hour cultured Vero E6 cell and incubated at 37°C+5%CO₂ for 48 hours. After incubation, SAR-CoV-2 antigen was detected by cell based ELISA. The cut off value was calculated according to formula as follow;

$$ODvaluecutpoint < \frac{(ODvalueof100TCID50virus - ODvalueofcellnegativecontrol) + ODvalueofcellnegativecontrol}{2}$$

The absorbance of sample containing neutralizing antibody should be less than cut point. Diluted serum with NT titer less than 10 were interpreted as negative and diluted serum with NT titer ≥ 20 were interpreted as positive, which contained SAR-CoV-2 specific neutralizing antibody.

Results

Samples were collected from 92 cases of RT-PCR confirmed COVID-19 patients who were admitted in Nakhon Pathom hospital, Siriraj hospital and Golden Jubilee Medical center during December 2020 to January 2021. There were 34 males and 52 females with aged between 20 to 63 years (mean 40.50±11.86 years). They were 47 asymptomatic infected individuals and 45 mild symptomatic patients who were treated with either Ivermectin+Zinc or Hydroxychloroquin+Durunavir/Ritonavir+Zinc (46 cases in each group).

Real-time RT-PCR was performed to detect SAR-CoV-2 RNA and level of viral load was inverse variation to RT-PCR cycle threshold (Ct). Samples from the 1st week showed a wide range of RT-PCR cycle threshold specific to E gene (from 11.76 to 39.43). These indicated the difference in duration of sample collection from each infected individual. The viral load was decreased in the following weeks as shown in the increasing of cycle threshold. The average of RT-PCR cycle threshold in the 1st, 2nd and 3rd week were 25.37±7.22, 29.61±5.65 and 33.08±4.66, respectively (figure 1). Changing rates of cycle threshold (ΔCt/day) in high viral load (Ct ≤ 30, at least once in the first two weeks) and low viral load samples were observed. Results showed that ΔCt/day of E gene in high viral load samples (n=62) were 1.47±1.98 within 1st week, 1.17±1.66 during 2nd week, 0.08±1.03 during 3rd week and 0.21±0.45 after 3rd week, while in low viral load samples (n=30) were 0.98±1.32 within 1st week, 0.37±1.49 during 2nd week,

0.23±1.18 during 3rd week and -0.11±0.50 after 3rd week. (figure 2). Changing rate of cycle threshold during 2nd week were significantly higher in high viral load samples than low viral load samples. Viral isolation was performed in Vero-E6 cell line. The positive result was observed by cytopathic effect and confirmed by immunofluorescent staining using SAR-CoV-2 nucleoprotein specific antibody. Only 18 cases (approximate 19%) were positive for viral culture and all of them contained high viral load (the average of RT-PCR cycle threshold was 19.69±5.27) (figure 3a). The Two cases with positive viral culture in the 2nd week (IDRA-065 and IDRA-092) were selected to observe NT-titer during 3 weeks after diagnosis and follow-up by micro-neutralization assay (figure 3b).

Antibody specific to SAR-CoV-2 RBD protein was detected in most cases (n=66). There were 28 males and 38 females with aged between 20 to 61 years (mean 41.11±11.72). In this 66 cases, there were 35 mild symptom patients and 31 asymptomatic infected individuals, and only 11 cases were positive for viral culture. The average of RT-PCR cycle threshold specific to E gene in the 1st, 2nd and 3rd week were 25.71±6.90 (range 11.76 to 39.34), 30.32±5.20 (range 15.72 to 39.54) and 33.90±3.99 (range 17.90 to 38.96), respectively. In 26 cases with non-detectable SAR-CoV-2 RBD protein specific antibody, were 6 males and 20 females with aged between 20 to 63 years (mean 38.96±12.29). There were 10 mild symptom patients and 16 asymptomatic infected individuals, and 7 cases were positive for viral culture. The average of RT-PCR cycle threshold specific to E gene in the 1st, 2nd and 3rd week were 24.35±8.25 (range 12.56 to 38.48), 27.70±6.49 (range 16.32 to 37.90) and 31.00±5.46 (range 18.79 to 37.05), respectively. These showed that some infected individuals might not develop specific antibody even though they had high viral load or mild symptom. Neutralization titer (NT titer) of case IDRA-065 and IDRA-092 were observed using micro-neutralization assay during 3 weeks after diagnosis and follow-up. These 2 cases were positive for viral isolation in the 2nd week, one of them (IDRA-092) had low level of neutralizing antibody in serum at the same time point and these neutralizing antibody level increased in the 3rd week and follow-up samples together with a drastic decline in viral load and negative viral culture, while the other (IDRA-065) showed high viral load and positive viral culture together with negative neutralizing antibody in the samples from the 2nd and 3rd week (figure 3b). This showed that failure in mounting the immune response in some individuals may result in persistent shedding of viable virus.

Discussion

Samples from the 1st week showed a wide range of cycle threshold (Ct) specific to E gene which indicated the difference in duration of sample collection from each infected individual. The viral load was decreased in the following weeks as shown in the increasing of cycle threshold (Ct). Changing rates of cycle threshold ($\Delta Ct/day$) during 2nd week were significantly higher in high viral load samples ($Ct \leq 30$, at least once in the first two weeks). Only 18 cases (approximate 19%) were positive for viral culture and all of them contained high viral load. These data indicate that most patients with persistent positive RT-PCR have low viral load and this does not indicated shedding of viable virus. Several studies also reported the persistence of viral RNA in recovered COVID-19 patients for long time which was not associated to viable viral shedding [5, 7, 9]. Specific antibody in serum was undetectable in 26 cases (approximate 28%) even though some of them had high viral load or had mild symptom. Undetectable specific antibody might be a result of immune response mediated through T cell response [12] and previous report showed that neutralizing antibody might be undetectable in mild symptom or asymptomatic COVID-19 patients [8, 13]. Neutralization titer (NT titer) was observed in 2 culture positive samples from the 2nd week, one of them had low level of neutralizing antibody in serum at the same time point and these neutralizing antibody level increased in the 3rd week and follow-up samples together with a drastic decline in viral load and negative viral culture. The other sample showed high viral load and positive viral culture together with negative neutralizing antibody in the samples from the 2nd and 3rd week. Failure in mounting the immune response in some individuals may result in persistent shedding of viable virus. High viral load and negative neutralizing antibody may be used as markers to identify such individuals.

Declarations

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Compliance with ethical standards

Conflict of interest

The authors declare no conflicts of interest.

Research involving human participants and/or animals

Human participants were involved in this research and this research project had been approved from Siriraj Institutional Review Board (*SIRB*), Faculty of Medicine, *Siriraj* Hospital, Mahidol University.

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Figures

Figure 1
Real time RT-PCR cycle threshold (Ct) of E gene (all 92 cases)

Figure 2

Real time RT-PCR cycle threshold (Ct) and changing rates of cycle threshold (Δ Ct/day) of E gene in high and low viral load groups (high viral load group: Ct ≤ 30, at least once in the first 2 weeks)

Figure 3

(a) Real time RT-PCR cycle threshold (Ct) of E gene in viral culture positive cases (black dot = Ct of each gene at the same time point of viral culture positive) (b) Real time RT-PCR cycle threshold (Ct) and NT titer of 2 culture positive cases (IDRA-065 and IDRA-092)