RESEARCH ARTICLE

Viral load and gastrointestinal inflammation in COVID-19 patients [version 1; peer review: awaiting peer review]

Aditya Riadi Syafei¹, Titong Sugihartono², Iswan Abbas Nusi², Poernomo Boedi Setiawan², Herry Purbayu², Ummi Maimunah², Ulfa Kholili², Budi Widodo², Husin Thamrin², Amie Vidyani², Hasan Maulahela³, Yoshio Yamaoka⁴, Muhammad Miftahussurur²,⁵

¹Department of Internal Medicine, Faculty of Medicine-Dr. Soetomo Teaching Hospital, Universitas Airlangga, Surabaya, Jawa Timur, 60286, Indonesia
²Gastroentero-Hepatology Division, Department of Internal Medicine, Faculty of Medicine-Dr. Soetomo Teaching Hospital, Universitas Airlangga, Surabaya, Jawa Timur, 60286, Indonesia
³Division of Gastroenterology, Department of Internal Medicine, Faculty of Medicine-Cipto Mangunkusumo Teaching Hospital, University of Indonesia, Jakarta, 10430, Indonesia
⁴Department of Environmental and Preventive Medicine, Oita University Faculty of Medicine, Yufu, Japan
⁵Helicobacter pylori and Microbiota Study Group, Institute of Tropical Disease, Universitas Airlangga, Surabaya, Jawa Timur, 60115, Indonesia

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Abstract

Background: ACE-2 receptors are well-known as binding receptors to spike protein of SARS-CoV-2 highly expressed in the gastrointestinal system. The Role of SARS-CoV-2 viral load and its effect on gut inflammation in COVID-19 patients are still not well-understood. This study aims to determine the impact of SARS-CoV-2 viral load on gastrointestinal inflammation.

Methods: A total of 44 inpatient subjects who fulfilled eligibility criteria were examined for cycle threshold values from nasopharyngeal swab samples collected from several nucleic levels based on fluorescence signal and calprotectin levels of stool samples using Calprotectin enzyme-linked immunosorbent assay (ELISA) kit.

Results: Of 44 subjects, 52.3% were male, with a median age of 52.5 years. Hypertension or diabetes was found in 26 patients. The median cycle threshold value was 31.3 with a value range of 10.9-40.0, median cycle threshold was significantly lower in subjects with comorbidity with \( P = 0.01 \). The median fecal calprotectin level was 42 μg/g with a value range of 5.1-1,393.7 μg/g, with median fecal calprotectin significantly higher in subjects with gastrointestinal symptoms with \( P = 0.008 \) with a relative risk (RR) of 5.5. There was a significant correlation between cycle threshold and fecal calprotectin in subjects with comorbidity with \( P < 0.05 \), a coefficient contingency of 0.414.

Conclusion: Subjects with comorbidity are prone to have higher viral loads paralleled with gastrointestinal inflammation. Subjects with
overt gastrointestinal manifestations had a five-fold higher degree of gut inflammation.

**Keywords**
COVID-19, cycle threshold, fecal calprotectin, gastrointestinal symptoms, infections
Introduction

Coronavirus Disease 2019 (COVID-19) has been declared a global pandemic by World Health Organization (WHO) since March 2020. COVID-19 is caused by infection of Coronavirus named Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), the genus of Betacoronavirus and family of Coronaviridae, similar to SARS-CoV and Middle East Respiratory Syndrome Coronavirus (MERS-CoV) that cause respiratory problems. The virus infects host cells that express Angiotensin-Converting Enzyme 2 (ACE-2) receptor, it is found higher in the lungs and gastrointestinal system. Infection of SARS-CoV-2 activates the innate immune system and then releases cytokines, chemokines, and pro-inflammatory mediators.

The current diagnostic test suggested by WHO is based on Nucleic Acid Amplification Test (NAAT), such as Reverse-Transcription Polymerase Chain Reaction (RT-PCR). The cycle threshold is the number of replication cycles required by the fluorescence signal to pass the threshold. Cycle threshold inversely correlated to quantitative viral loads detected in the sample, although PCR testing does not distinguish live viruses from nonviable viruses. Several studies showed the clinical utility of cycle threshold value and high viral load correlated with disease severity, but other studies show no correlation in different samples settings. Clinical symptoms of COVID-19 are fever, cough, respiratory symptoms, and currently, extra-pulmonary clinical manifestations, including gastrointestinal, have been reported to vary in more than 20% of hospitalized patients. One study in 2020 showed the presence of the SARS-CoV-2 genome in stool samples. This proves that the SARS-CoV-2 infection in the gastrointestinal tract is thought to be caused by the expression of ACE-2 in the digestive tract. Transmembrane Serine Protease 2 (TMPRSS2) is expressed including in gastrointestinal epithelium, that facilitates cell entry by priming of the spike protein.

Several studies found virus RNA in stool specimens and anal swabs, however, it remains unclear when the virus starts shedding in the gastrointestinal tract and takes a longer duration of viral shedding than in the respiratory tract. Gastrointestinal manifestations in SARS-CoV-2 infected patients provide clues whether inflammation occurs in gastrointestinal tissue. Fecal calprotectin, a protein released by neutrophils and used as a direct marker of mucosal inflammation of any etiology, sensitive and non-invasive biomarker in mucosal inflammation. Diarrhea in cases of viral infection can naturally be inflammatory or non-inflammatory, based on the research data, it includes the results of non-inflammatory mechanisms. Another study in 2021 showed an increase in fecal calprotectin levels in 66.7% of COVID-19 patients in asymptomatic gastrointestinal tract patients.

The gastrointestinal system in SARS-CoV-2 infected patients occur gut microbiome alteration characterized by a decrease in diversity with shifting toward pathogenic bacteria and away from beneficial symbionts. The virus enters cells by recognizing receptor ACE-2 to invade the host cell via TMPRSS2 and initiate an immune response that results in increased gut permeability and dysbiosis. Gut dysbiosis and ACE-2 impairment lead to a leaky gut. In a viral infection, high levels of circulating pro-inflammatory cytokines can alter the gut microbiome and disturb intestinal integrity. Increased inflammation in the intestine leads to a leaky gut allowing bacterial antigens and toxins to translocate to the systemic circulation, leading to worsening clinical outcomes in COVID-19 patients.

Studies analyzing fecal calprotectin levels in SARS-CoV-2 infection are relatively still limited. Some studies mention the association between cycle threshold values and disease severity, while other studies show significant fecal calprotectin increase results in COVID-19 patients, but studies on the association between the two have yet to be understood. We determined a correlation between the cycle threshold value of nasopharyngeal swab and fecal calprotectin in COVID-19 patients.

Methods

Study design and participants

This research was an observational analytic study with a cross-sectional design. We conducted consecutive sampling at Dr. Soetomo Teaching Hospital Surabaya from 1 September to 30 November 2020. Since there was no prior study of these specific subjects we decided to count the minimum sample size using infinite population formula. We analyzed 44 samples from non-ICU that meet this research eligibility criterion. Inclusion criteria were age 18 or above, a confirmed case of COVID-19, and signed participants agreement of this study. Exclusion criteria were the patient’s refusal to participate in this study, gastrointestinal malignancies, liver cirrhosis, End-Stage Renal Disease, and Inflammatory Bowel Disease. Written informed consent was obtained from all patients and the research protocol approved by Faculty of Medicine, Universitas Airlangga-Dr. Soetomo Teaching Hospital Surabaya, Indonesia health research ethics committee with certificate number 0065/KEPK/IX/2020. Patients confirmed COVID-19 based on PCR results from nasopharyngeal swab specimens. The RT-PCR cycle threshold value was measured from an analytical phase of a nasopharyngeal swab specimen using a nucleocapsid (N) gene target with a negative value >40. Analytical phase was conducted in biosafety level 2 facility in close system with automated process, using rt-PCR kit Biocov, Acon and light cycler Roche or Gentier (China). Cycle threshold value data were collected from clinical microbiologists at The Department of Clinical
Microbiology Faculty of Medicine Universitas Airlangga, Surabaya. Fecal calprotectin level is calprotectin measured in the patient’s feces sample. Stool preparations are taken by the nurse or by the patient. Stool with a minimum of five grams was analyzed using the PhiCal© Calprotectin enzyme-linked immunosorbent assay (ELISA) kit (Immundiagnostik AG, Stubenwald-Allee 8a, D-64625 Bensheim). Calprotectin remained stable in feces for six days with a normal reference value of <50 μg/g. We took 44 samples that were eligible for this study and analyzed all samples as subjects fulfilled all research variables.

Statistical analysis
Data were analyzed using the IBM SPSS Statistics version 25 (IBM Corp., USA). Demographic data and clinical characteristics are presented descriptively, frequency and percent for categorical data types (nominal and ordinal), while mean, standard deviation, median, and minimum-maximum for continuous data (interval and ratio). The independent variable is the cycle threshold value as numerical data (ratio and interval). The dependent variable is fecal calprotectin level as numerical data (ratio and nominal). Correlational numerical analytic tests carried out the association between variables in this study. The correlation test was performed using Pearson. The P-value that is considered statistically significant was <0.05 and the confidence interval (CI) was 95%.

Results
Characteristics of subjects
Subjects based on gender were male with 52.3% and female was 47.7%. The median age of subjects was 52.5 years with the youngest age of 18 years and the oldest of 65 years. The highest number of subjects was based on a range of age 51-60 years at 29.54%. Eighteen subjects were without comorbidity (40.9%) and the highest comorbidity in subjects was diabetes mellitus and hypertension in 14 patients (31.8%), followed by diabetes mellitus 18.2% and hypertension 9.1%. The characteristics of subjects based on clinical manifestations were cough 75%, fever 70.5%, shortness of breath 68.2%, and anosmia 9.1%. The characteristics of subjects based on gastrointestinal manifestations were anorexia 63.6%, nausea 38.6%, diarrhea 31.8%, vomiting 29.5%, and abdominal pain 11.4%. Mean symptom onset was 4.02 ± 2.64 days with a median of three days. The shortest onset was one day and the longest onset was 14 days.

Table 1 shows median systolic and diastolic blood pressures of 130 and 80 mmHg, the median pulse of 88 times per minute, median respiratory rate of 21.5 times per minute, median axillary temperature of 36.7°C, and peripheral oxygen saturation of 97%. Laboratory characteristics of subjects showed a median lymphocyte value of 1,295/mm³ slightly below the normal range of 1,300-4,000/mm³, the median neutrophil-lymphocyte ratio was 4.9348, median ferritin was 588.4 μg/L, and median D-dimer was 1,150 μg/L.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Total (n = 44)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median</td>
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<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>130</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>80</td>
</tr>
<tr>
<td>Pulse (times per minute)</td>
<td>88</td>
</tr>
<tr>
<td>Respiratory rate (times per minute)</td>
<td>21.5</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>36.7</td>
</tr>
<tr>
<td>Peripheral oxygen saturation (%)</td>
<td>97</td>
</tr>
<tr>
<td>Hemoglobin (g/dl)</td>
<td>12.4</td>
</tr>
<tr>
<td>Leukocytes (/mm³)</td>
<td>9,280</td>
</tr>
<tr>
<td>Absolute neutrophils (/mm³)</td>
<td>6,930</td>
</tr>
<tr>
<td>Absolute lymphocyte (/mm³)</td>
<td>1,295</td>
</tr>
<tr>
<td>Neutrophil-lymphocyte ratio</td>
<td>4.9348</td>
</tr>
<tr>
<td>Platelets (/mm³)</td>
<td>330,000</td>
</tr>
<tr>
<td>CRP (mg/dl)</td>
<td>0.8</td>
</tr>
<tr>
<td>Ferritin (μg/L)</td>
<td>588,400</td>
</tr>
<tr>
<td>D-dimer (ng/ml)</td>
<td>1,150</td>
</tr>
<tr>
<td>Procalcitonin (μg/L)</td>
<td>0.09</td>
</tr>
</tbody>
</table>

CRP: C reactive protein.
Table 2. Fecal calprotectin levels in subjects with gastrointestinal symptoms.

<table>
<thead>
<tr>
<th>Subjects (n = 44)</th>
<th>Fecal calprotectin (μg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
</tr>
<tr>
<td>With gastrointestinal symptoms</td>
<td>170.65 ± 280.64</td>
</tr>
<tr>
<td>Without gastrointestinal symptoms</td>
<td>25.64 ± 15.92</td>
</tr>
<tr>
<td>With diarrhea</td>
<td>298.0 ± 365.47</td>
</tr>
<tr>
<td>Without diarrhea</td>
<td>43.55 ± 67.88</td>
</tr>
</tbody>
</table>

*Mann-Whitney test; SD: Standard Deviation.

Nasopharyngeal swab RT-PCR cycle threshold value in subjects

The measurement of cycle threshold value was obtained based on fluorescence with RT-PCR technique used to detect SARS-CoV-2 genes in upper respiratory tract specimens of subjects. This value is obtained based on a real-time amplification curve based on signal changes and then determines the quantitative detection of SARS-CoV-2 at the nucleic acid level. The mean cycle threshold value for all subjects was 30.33 ± 6.07 with a median of 31.31, a minimum value of 10.09, and a maximum of 40.00. The median cycle threshold value in subjects with comorbidity was 30.4 significantly lower (P = 0.01). Comorbidity consists of diabetes mellitus, hypertension, or a combination of these. Distribution of subjects with gastrointestinal symptoms and subjects with diarrhea symptoms did not significantly differ in cycle threshold value (Mann-Whitney test, P = 0.921, and P = 0.521, respectively). Distribution of cycle threshold value in subjects group based on WHO severity classification also did not show a significant difference between mild, moderate, severe, or critical (the Kruskal-Wallis test, P = 0.436).

Fecal calprotectin levels in subjects

Based on the kit used in the laboratory, fecal calprotectin cut-off value <50 μg/g was considered negative. In this study, 26 subjects (59.1%) with fecal calprotectin levels <50 μg/g, subjects with fecal calprotectin levels ≥50 μg/g were 18 patients (40.9%). The mean fecal calprotectin level in all subjects is 124.51 ± 240.54 μg/g with a median of 42 μg/g and a minimum value of 5.1 μg/g and a maximum of 1,393.7 μg/g. Table 2 showed the results of the Mann-Whitney test, statistically significant differences in fecal calprotectin increase in the group of subjects with gastrointestinal symptoms (61.70 vs 21.55 μg/g, P = 0.008, respectively). This study also found that fecal calprotectin correlates with hemoglobin levels with P < 0.05 and a coefficient correlation of -0.307. The median value of fecal calprotectin levels was found higher in groups of subjects with symptoms of diarrhea than in subjects without symptoms of diarrhea (143.45 vs 24.4 μg/g, P = 0.0001, respectively). Subjects with symptoms of diarrhea tend to have fecal calprotectin levels ≥50 μg/g with a relative risk (RR) of 5.57 (95% CI; IQR 2.47-12.56) with P < 0.0001 in the Chi-Square test. This study also found that fecal calprotectin correlates with D-dimer in subjects with symptoms of diarrhea with P = 0.024 and a coefficient correlation of 0.598 (normal reference of D-dimer <500 ng/ml).

Cycle threshold and fecal calprotectin

The non-parametric Spearman correlation test showed no significant correlation between the cycle threshold value and fecal calprotectin levels (P = 0.613; r = 0.078) in this study. Spearman’s non-parametric test showed no statistically significant relationship between the cycle threshold value and fecal calprotectin levels in subjects with gastrointestinal symptoms and diarrhea symptoms (P = 0.353 and P = 0.817, respectively). However, in subjects with comorbidity, based on Fisher’s exact test there was a sufficient correlation between cycle threshold value with fecal calprotectin cut-off of ≥50 μg/g with P < 0.05, contingency coefficient of 0.414.

Discussion

This study found that viral load correlates with gastrointestinal inflammation in the population with comorbidity. This study also showed patients with comorbidity have higher viral loads than subjects without comorbidity. These findings suggest that comorbidity becomes an independent risk factor for gastrointestinal inflammation and an infection risk factor. Studies showed that people with comorbidity are at higher risk of infection and have more severe disease manifestations. Several studies suggested that patients with comorbidity had compromised immune systems to infection. This study found that subjects with comorbidity had a lower cycle threshold than subjects without comorbidity suggesting that patients with comorbidity are prone to have more viral load and inadequate immune response to infection. A study in 2020 found that the risk of infection in a population with comorbidities such as diabetes mellitus or hypertension is associated with the up-regulation of ACE-2. Another study also showed a risk of mortality relatively higher in populations with comorbidity.
Widely known SARS-CoV-2 is attached to ACE-2 receptors in the respiratory or gastrointestinal system. Up-regulation of ACE-2 receptors in comorbidity population, attached with the virus with inappropriate immune response causing ineffective defense mechanism against the virus and replicating at higher levels. A study in 2020 stated that patients with comorbidity had a decrease in gut microbiome diversity. The gut microbiome had several benefits as normal flora in gastrointestinal systems, such as a defense mechanism against pathologic entities. Several pathomechanisms disrupt gastrointestinal mucosal homeostasis. This study found in subjects with diarrhea symptoms, fecal calprotectin correlates with D-dimer. COVID-19 that occurs hypercoagulable state, caused by endothelial dysfunction that causes fibrin to degrade and release D-dimer protein component in the infected patient was found significantly increased. This pro-thrombotic state could induce tissue ischemia, including intestinal mucosa layer, activation of a local gut immune response, and induce calprotectin release. This study also found that fecal calprotectin negatively correlates with hemoglobin. This founding shed a new perspective on the role of thrombosis and the consequences of intestinal damage. Gastrointestinal inflammation correlates with more severe anemia due to colitis or micro gastrointestinal bleeding need further study to investigate. Hyper-inflammatory state in COVID-19 not only causes alteration in gastrointestinal homeostasis led to inflammation but also alters the metabolism of iron by producing hepcidin, in response to IL-6 activity, that causes reduced transferrin and inhibits iron absorption in the intestinal ultimately causing iron deficiency. The limited understanding of gastrointestinal inflammation and its effect on systemic iron metabolism needs further investigation.

This study also found fecal calprotectin potential as a non-invasive biomarker to determine disruption of gastrointestinal mucosal homeostasis, whether due to direct viral infection on gut epithelial cells or secondary to systemic inflammation response. This study also found that gut inflammation happened more severely in subjects with an overt gastrointestinal manifestation such as abdominal pain, diarrhea, nausea, vomiting, and anorexia. One study suggests viral infect enteroids, disrupt intestinal permeability resulting in enterocyte dysfunction. Studies in 2020 also support these findings of significantly increasing fecal calprotectin in patients who suffered diarrhea. One study suggests that symptoms may be direct mucosal pathology of viral infection or secondary to virus-induced inflammation, in turn, due to the entry of inflammatory cells into intestinal mucosa, including neutrophils and lymphocytes, and thus disruption of gut microbiota. This also found higher viral loads in stool specimens in subjects with diarrhea symptoms. One study in 2021 found specific Immunoglobulin A (IgA) of Receptor Binding Domain (RBD) specific to SARS-CoV-2 in stool, this suggesting that immune response to inflammation in a gut system can be primary or secondary from a systemic response.

Several studies found that viral clearance in the gut system takes longer than in the respiratory system. Virus clearance in the gut system varies up to several days to weeks after seroconversion from upper respiratory samples. A study conducted in 2020 stated that patients with gastrointestinal symptoms have more extended duration virus clearance and are more likely to have positive stool tests. This suggests the potential risk of fecal-oral transmission and raises the question of whether this prolonged delayed clearance in the gut system causes persistent gastrointestinal inflammation or becomes one of the long COVID-19 manifestations that needs further investigation.

There were several limitations of this study. The monocentric study with a small sample of this study might not be enough to represent the general population. Therefore, further investigation with more samples collected might be needed. In addition, this study did not consider the time of sample collecting and did not analyze with cycle threshold from anal swabs or stool specimens.

**Conclusion**

There were significantly higher viral loads in subjects with comorbidity in this study. Subjects with overt gastrointestinal manifestations had a five-fold higher degree of gut inflammation. Viral loads and gut inflammation were correlated in subjects with comorbidity.

**Data availability**


This project contains the following underlying data:

- The raw material data contain much information about general biodata patients, the observational result of vital signs, head/neck, thoracic, abdominal, and extremity.

- In addition, the data include Peripheral Complete Blood Count, Inflammation markers, CT value to see the viral load, and the fecal calprotectin
Data are available under the terms of the Creative Commons Zero “No rights reserved” data waiver (CC0 1.0 Public domain dedication).

Author contribution
Syafei, AR: Conceptualization, Methodology, Writing—original draft preparation; Writing—review and editing; Sugihartono, T: Conceptualization, Writing—original draft preparation, Writing—review and editing; Nusi, IA: Methodology, Resource; Setiawan, PB: Data accuration, Validation; Purbayu, H: Methodology, Writing—review and editing; Maimunah, U: Methodology, Writing—original draft preparation; Kholili, U: Writing—review and editing, Validation; Widodo, B: Conceptualization, Writing—review and editing; Thamrin, H: Methodology, Resource; Vidyani, A: Data curration, Validation; Maulahela, H: Data accuration, Investigation; Yamaoka, Y: Data Accuration, Supervision; Mitfaussurur, M: Conceptualization, Supervision, Writing—original draft preparation.

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References


Author contribution
Syafei, AR: Conceptualization, Methodology, Writing—original draft preparation; Writing—review and editing; Sugihartono, T: Conceptualization, Writing—original draft preparation, Writing—review and editing; Nusi, IA: Methodology, Resource; Setiawan, PB: Data accuration, Validation; Purbayu, H: Methodology, Writing—review and editing; Maimunah, U: Methodology, Writing—original draft preparation; Kholili, U: Writing—review and editing, Validation; Widodo, B: Conceptualization, Writing—review and editing; Thamrin, H: Methodology, Resource; Vidyani, A: Data curration, Validation; Maulahela, H: Data accuration, Investigation; Yamaoka, Y: Data Accuration, Supervision; Mitfaussurur, M: Conceptualization, Supervision, Writing—original draft preparation.

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