Research Article

Intestinal Protozoan Infections in COVID-19 Patients and Isolation of *Cryptosporidium parvum var 1.* from Recurrent COVID-19 Patients

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Abstract | Intestinal protozoans can be activated due to the weakening of the immune system and cause diagnostic difficulties and death of patients due to symptoms overlapping with COVID-19 disease. It was aimed to investigate the distribution of intestinal protozoan infections in COVID-19 patients and the relationship between these protozoans with clinical symptoms of COVID-19 disease. COVID-19 patients included in the study were classified according to their clinical symptoms as mild, moderate and severe clinical symptoms. ELISA tests were used to determine the $CD4^+$T and $CD8^+$T cells values of 148 COVID-19 patients. Microscopy, ELISA, PCR, real-time PCR and DNA sequence analyses were used to identify intestinal protozoan such as *Blastocystis spp*., *C. parvum*, *E. histolytica* and *G. duodenalis*. While intestinal protozoans were detected 22.3% (33/148) of COVID-19 patients, intestinal protozoans were not detected 77.7% (115/148) in this study. *Blastocystis spp.* was frequently detected 39.4% (13/33) followed by *C. parvum* 27.3% (9/33) then *G. duodenalis* 21.2% (7/33) and *E. histolytica* 12.1% (4/33) among COVID-19 patients. While all of these intestinal protozoans were detected in COVID-19 patients with severe clinical symptoms, *Blastocystis spp*. and *G. duodenalis* were detected in COVID-19 patients with moderate clinical symptoms. However, only *Blastocystis spp*. was detected in COVID-19 patients with mild clinical symptoms. In addition, the genetic distance value of *C. parvum var 1* isolated from recurrent COVID-19 patients in the study was found to be between 0.05 (27/517) and 0.07 (37/310) in 120 bp-200 bp nucleotide. Our study shows that in addition to diseases such as diabetes and hypertension, intestinal protozoan infections can be a comorbidity with COVID-19 disease and increase the mortality rate of COVID-19 disease. In addition, our study shows that *C. parvum var 1* may be the cause in recurrent COVID-19 disease. Therefore, it is useful to consider intestinal protozoan infections in the follow-up and treatment processes of COVID-19 patients.

Keywords | COVID-19 disease, intestinal protozoans, *C. parvum var 1*, $CD4^+$ and $CD8^+$ T cells

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INTRODUCTION

Coronavirus Disease (COVID-19) emerged in China at the end of 2019 and this disease is caused by severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) (Lai et al., 2020). According to the data of World Health Organization (WHO), as of 27 January 2023, there have been 752.517.552 confirmed cases of COVID-19, including 6.804.491 deaths in the worldwide (who.int., 2023). The 17.004.677 cases (101.419 deaths) of these COVID-19 cases reported worldwide were reported from Turkey, located on the 26°-45°E meridians and 36°-42°N parallels (who.int., 2023). The COVID-19 pandemic continues to be an important

public health threat in Turkey as well as over the world. The intestinal protozoan infections are one of the important health problems in developing countries such as Turkey, despite the continuous efforts and programmes organized by WHO (Barazesh et al., 2015). Intestinal protozoans such as *Cryptosporidium parvum (C. parvum)*, *Cystoisopora belli (C. belli)*, and *Cyclospora cayetanensis (C. cayetanesis)* are generally more common in immunocompromised individuals (Laksemi et al., 2019). However, various species of enteric intestinal protozoan such as *Entamoeba histolytica* (*E. histolytica*), *Giardia duodenalis* (*G. duodenalis*), *Dientamoeba fragilis* (*D. fragilis*) are associated with diarrheal illnesses in humans, and these protozoans cause severe debilitating diseases, especially in immunocompromised individuals (Fletcher et al., 2012). It has been reported that the rates of diseases and deaths due to intestinal protozoans are high in developed and developing countries, and these diseases adversely affected the economies of the countries (Fletcher et al., 2012).

The incidence and severity of intestinal protozoans may vary due to the daily increase in the number of immunocompromised individuals, increased organ transplant surgery, immunocompromised drugs, radition therapy, and the COVID-19 disease. This disease, which emerged in last three years, has changed the epidemiology, therapy and pathology of many diseases due to its immunocompromise feature. However, since there is no study investigating the risk of contracting intestinal protozoans in COVID-19 patients with weakened immune response, we investigated the incidence of intestinal protozoans in COVID-19 patients in this study. The species of intestinal protozoans isolated from the COVID-19 patients were determined by DNA sequence and the relationship between intestinal protozoans and clinical features (mild, moderate, severe clinical symptoms) of COVID-19 disease was investigated.

MATERIALS AND METHODS

Study Population

This study was carried out between February 2021 to March 2022 at the Aksaray University Training and Research Hospital and the study was approved by Necmettin Erbakan University Clinical Research Ethics Committee (Protocol No: 08.01.2021-2021/2982). A total of 148 patients with clinical symptoms of fever, cough, shortness of breath, chills, abdominal pain, diarrhea and confirmed to be COVID-19 by real-time PCR were included in this study. On the other hand, COVID-19 patients with asymptomatic clinical symptoms and no diarrhea were not included in this study.

COVID-19 patients included in the study were categories according to WHO criteria as mild, moderate and severe according to their clinical characteristics. COVID-19 patients with frequent fever, cough and fatigue were defined as mild clinical symptoms, patients with difficulty in breathing and mild pneumonia were defined as moderate clinical symptoms, patients with severe pneumonia, organ failure and dyspnea were defined as severe clinical symptoms in this study. Of the COVID-19 patients, 32.4% (48/148) had mild clinical symptoms, 27.1 (40/148) had moderate and 40.5 (60/148) had severe clinical symptoms. While chronic disease comorbidity was detected in 32.4% (48/148) (12.2% diabetes, 9.5% hypertension, 7.4% cardiovascular and 3.3% chronic liver disease) of 148 COVID-19 patients included in the study, no chronic disease was detected in 67.6% (100/148) COVID-19 patients. The age, gender, chronic diseases, and categories of the patients included in the study were evaluated and shown in Table 1. A total of 148 COVID-19 patients included in this cross-sectional study 63 (42.6%) were female and 85 (57.4%) were male ($p \leq 0.05$). The age of the patients included in the study was between the age of 44-82 years with median of ±68.7 years. The mean age of the male patients was ±69.5 and the mean age of the female patients was ±67.5 in this study. It wasn't find significant difference between the ages of male patients and female patients ($p > 0.05$). The heathy control group of the study consisted of 148 peope who were not diagnosed with COVID-19 disease and they were not have a chronic disease.

Table 1: The age, gender, chronic diseases, and categories of the COVID-19 patients.

Characteristics	All patient (n=148, %)
Clinical symptoms	
Mild	32.4
Moderate	44953
Severe	40.5
Age	
Female age	±68.7
Male age	±69.5
Gender	
Female	42.6
Male	57.4
Chronic medical illness	
Diabetes	12.2
Hypertension	9.5
Cardiovascular diseases	7.4
Chronic liver disease	3.3
No chronic medical illness	67.6

$CD4^+$ And $CD8^+$ T Cell Values Of Covid-19 Patients

ELISA tests were used to determine the $CD4^+$T and

$CD8^+$T cell values of patients diagnosed with COVID-19 and the intestinal parasites in these patients. Two cc blood samples were taken from 148 patients with COVID-19 patients and healthy control individuals. After the blood samples were centrifuged at 1000 g for 15 min, the serum samples were used in the study. To measure the $CD4^+$T cell and $CD8^+$T cell values of patients with COVID-19, the $CD4^+$T cell human ELISA and $CD8^+$T cell human ELISA (Aviva Systems Biology, USA) commercial kit was used according to the manufacturer's instructions. The 50 µL of the stop solution was added according to the kit's instructions for use and the reactions in the wells turned yellow. The optical density (OD) absorbance was provided at 450 nm by an automatic ELISA reader with a standard microplate reader within 5 minutes of stopping the reaction. The results of the assays were calculated the relative OD_{450} for each test based on the ELISA kits recommendations.

Investigaion Of Intestinal Protozoans In Covid-19 Patients

Microscopic Method: In order to investigate intestinal protozoan, stool samples were taken from COVID-19 patients and healthy control individuals. The wet slides were prepared using normal saline and iodine from fresh stool samples and examined under a light microscope. Intestinal protozoans were investigated in stool samples taken from COVID-19 patients using modified acid-fast staining and trichome staining methods.

Elisa Method: Intestinal protozoans such as *Blastocystis spp.*, *C. parvum*, *E. histolytica*, and *G. duodenalis* (Hand-picked Antibodies, Germany) were investigated using ELISA stool human kit according to the manufacturer's instructions and the OD values were evaluated at 450 nm using an automatic ELISA reader. Those with an average OD values of samples ≥1.0 were considered positive, and those with an average OD value of ≤ 0.15 were considered negative according to the results of ELISA reader.

Real-Time Pcr Method: DNA samples were isolated from stool samples taken from COVID-19 patients using QIAamp DNA mini stool kits (Qiagen, Hilden, Germany). The real-time PCR analysis was performed using different primers and reactions for each of the *Blastocystis spp.*, *C. parvum*, *E. histolytica*, and *G. duodenalis*. The gene regions targeted and the primer-probes used to identify these intestinal parasites have been shown in Table 2. All primers and probe sequences used in this study were studied according to the previously published and used procedure of Menu et al. (Menu et al., 2021).

The real-time PCR reactions were conducted using a total volume of 20 µL containing 1X BioRad real-time pcr master mix (Life Science, Marnesla-Coquette, France), 10 µM each primer, 5 µM probes, 3.5 µL of distilled water, and 5 µL of DNA. The real-time PCR method were analyzed using a BioRad CFX96TM real-time PCR detection system (Life Science, Marnes-la-Coquette, France). The real-time PCR reactions and amplification thermal-cycler programs have shown in table 1. In real-time PCR reaction amplification results, those with a Ct value below 30 were considered positive, and those with a Ct value above 28 were considered negative.

PCR Method: The gene regions specific of *Blastocystis spp*, *C. parvum*, *E. histolytica* and *G. duodenalis* protozoans were targeted and primers were designed for PCR method (Ramirez et al., 2014; Xiao et al., 2000; Qader et al., 2011; Som et al., 2000) (Table 3). PCR reactions were prepared for each protozoan and each reaction was amplified for analysis in a different thermal cycler protocol. Reactions containing 2X PCR reaction buffer, 1.5 mM of $MgCl_2$, 0.75 µM forward primer, 0.75 µM reverse primer, 1 unit Taq polymerase, 5 µL DNA sample and 4.5 µL distiled water were prepared for each PCR reaction a 25 ml final volume.

The first step of the thermal cycler programme was at 94°C for 10 min while the last step was at 72°C for 10 min. The target gene regions of protozoans, primers and thermal cycler protocol has shown in table 3. The PCR amplification products were analysed by electrophoresis method using 1.5% agarose gel stained with 0.5 µg/mL ethidium bromide stain.

DNA Sequencing And Phylogenetc Method: After the PCR products were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany), they were analyzed using the Big Dye Terminator DNA sequecing kit and an ABI Prism 310TM Genetic Analyzer (Applied Biosystems, California, USA). The double stranded sequences obtained as a result of DNA sequence analysis were arranged and aligned using the BioEdit program, v5.0.9 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The cross-comparison of the nucleotide sequences of isolated intestinal protozoan in the study was made using the neighbor-joining (NJ) method for phylogenetic analysis. A phylogenetic tree was constructed from the nucleotide sequences using the ClustalW multi-alignment software and the Tamura 3-parameter model in MEGA soft-ware version 6.05 in the study. The nuclotide sequences of intestinal protozoans were compared with *Blastocystis spp.* (ATCC 50177™), *C. parvum* (ATCC-PRA 67DQ™), *G. duodenalis* (ATCC-PRA 242™), *E. histolytica* (ATCC 30459™) strains obtained from ATCC.

Table 2: The primer-probe list used to identify intetsinal protozoans in the real-time PCR method.

Target Organism	Gene Region	Primer/Probe (5'-3')	Thermal-cycler condition	Ref
Blastocytis spp.	18srRNA	F: GGAGAGGGAGCCTGAGAGAT	95°C 10 min, 35 cycles,	Menu et.al.
		R: AACATTGTTCCGCATTGTGA	(95°C 15 s, 56°C 30 s, 72°C 30 s)	
		P: ATCCTGACACAGGGAGGTAGTG-VIC	72°C 10 min	
C. parvum	18srRNA	F: CATGGATAACCGTGGTAAT	94°C 10 min,35 cycles	Menu et.al.
		R: TACCCTACCGTCTAAAGCTG	(94°C 10 s, 54°C 30 s, 72°C 10 s)	
		P: ATCACATTAAATGT-VIC	72°C 10 min	
		P: CAATTCTAGCCGCTTAT-FAM	72°C 10 min	
E. histolytica	16srRNA	F: ATTGTCGTGGATCCTAACTCA	95°C 10 min, 35 cycles	Menu et.al.
		R: GCGGACCGCTCATTATAACA	(95°C 30 S, 60°C 30 s, 72°C 30 s)	
		P: FAM-TCATTGAATGAATTGGCCAT-TT-TAMRA	72°C 10 min	
G. duadenalis	SsuRNA	F: GACGGCTCAGGACAACGGTT	95°C 10 min, 35 cycles	Menu et.al.
		R: TTGCCAGCGGTGTCCG	(95°C 30 S, 59°C 30 s, 72°C 30 s)	
		P: FAM-CCCGCGGCG-GTCCCTGCTAG-TAMRA	72°C 10 min	

Table 3: Primers and thermalcyler program used to identify *Blastocystis spp*., *C. parvum*, *E. histolytica* and *G. duodenalis* in this study.

Target Organism	Gene	Primer/Probe (5'-3')	Thermal-cycler condition	Reference
Blastocystis spp.	SSuRD-NA	R: ATCTGGTTGATCCTGCCAGT	95°C 10 min, 35 cycles	Ramirez et al.
		F: GAGCTTTTTAACTGCAACAACG	(95°C 30s, 58°C 60 s,72°C 30 s) 72°C 10 min	
C. parvum	Cowp	R: GTAGATAATGGAAGAGATTGTG	95°C 10 min, 30 cycles	Xiao et al.
		F: GACTGAAATACAGGCAT-TATCTTG	(95°C 30s, 65°C 180 s,72°C 30 s) 72°C 10 min	
E. histolytica	ITS2	R: AGGTGAACCTGCGGAAGGAT-CATTA	95°C 10 min, 35 cycles	Som et al.
		F:TCATTCGCCATTACTTAAGAAAT-CATTGTT	(95°C 30s, 50°C 60 s,72°C 30 s) 72°C 10 min	
G. duadenalis	GDH2	R: CAGTACAACTCTGCTCTCGG	94°C 10 min, 35 cycles	Qader et al.
		F: GTTGTCCTTGCATCTCC	(94°C 30s, 61°C 60 s,72°C 30 s) 72°C 10 min	

Statistical Analyses

All data were entered to computer and anayased with Statistical Package for the Social Science v.22.0 software (SPSS Inc., Chicago, USA) and p-value were used for comparisons age, gender, clinical symptoms and intestinal protozoans. Mann-Whitney U test and Fisher's exact test were used to determine the distribution of intestinal protozoans detected in COVID-19 patients. Categorization of COVID-19 patients and intestinal protozoan were detected in these patients are defined as percentage data. Data having p-value<0.05 were considered to be statistically significant.

RESULTS

In a healthy individual, $CD4^+$T cell counts should be 500 ≤ $CD4^+$T cell ≤ 1200mm^3 and $CD8^+$T cell counts should be 150 ≤ $CD4^+$T cell ≤ 1000/mm^3. In addition, if the $CD4^+$T cell/$CD8^+$T cell >1, the immune system is strong, and if this ratio is less than 1 $CD4^+$T cell/$CD8^+$T cell <1, the immune system is weak. The $CD4^+$T cell and $CD8^+$T cell values were found in the normal range in the healthy control group in our study. In all of severe COVID-19 patients, the $CD4^+$T cell counts were below 500 ($CD4^+$T cell <500 cell/mm^3) and the $CD8^+$T cell counts were below 150 ($CD8^+$T cell <150 cell/mm^3). The average of $CD4^+$T cell was 300 cell/mm^3, the average of $CD8^+$T cell was 102

cell/mm^3, and the ratio of CD4$^+$T/CD8$^+$T was found to be less than 1 in all of severe COVID-19 patients (CD4$^+$T/CD8$^+$T<1 cell/mm^3). It was observed that the CD4$^+$T cell and CD8$^+$T cell values of these patients were significantly decreased ($p<0.05$). However, CD4$^+$T cell and CD8$^+$T cell values of mild and moderate COVID-19 patients were found to be between normal values. The CD4$^+$T/CD8$^+$T ratios of these patients were found to be greater than 1 (CD4$^+$T/CD8$^+$T >1 cell/mm^3). The results of CD4$^+$T and CD8$^+$T values according to the clinical symptoms of COVID-19 patients has been shown in Table 1.

All stool samples taken from the COVID-19 patients were analysed by microscopic method. While intestinal protozoans were detected in 16.9%(25/148) of stool samples, intestinal protozoans were not detected in 83.1% (123/148). It was determined that 36% (9/25) *Blastocystis spp*., 32% (8/25) *C. parvum*, 24% (6/25) *G. duodenalis* and 8% (2/25) *E. histolytica* of this patients by microscopic method (Table 4).

Table 4: The positive/negative value of patients by diagnostic methods used to identify intestinal protozoans and distribution of intestinal protozoans.

All patient (n=148, %)	Microspic	ELISA	RT-PCR	PCR
Positive value	16.9	17.6	22.3	20.3
Negative value	83.1	82.4	77.7	79.7
Protozoans species				
Blastocystis spp.	36	34.6	39.4	40
C. parvum	32	30.8	27.3	26.7
G. duodenalis	24	23.1	21.2	20
E. histolytica	8	11.5	12.1	13.3

According to the results of the ELISA method, while intestinal protozoans were detected in 17.6% (26/148) of the COVID-19 patients, no parasites were detected in 82.4% (122/148) of them (Table 4). According to the results of this diagnostic method, 34.6% (9/26) *Blastocystis spp*., 30.8% (8/26) *C. parvum*, 23.1% (6/26) *G. duodenalis* and 11.5% (3/26) *E. histolytica* intestinal protozoans were found in COVID-19 patients (Table 4).

While intestinal protozoans were detected in 22.3% (33/148) of COVID-19 patients, intestinal protozoans were not detected in 77.7% (115/148) using by real-time PCR in this study. With the real-time PCR method, *Blastocystis spp.* was frequently detected 39.4% (13/33) followed by *C. parvum* 27.3% (9/33) then *G. duodenalis* 21.2% (7/33) and *E. histolytica* 12.1% (4/33) in COVID-19 patients (Table 4).

According to the results of the PCR method, we detected intestinal protozoans in 20.3% (30/148) of the COVID-19 patients, while we could not find intestinal protozoans in 79.7% (118/148) of the patients. Using PCR method, 40% (12/30) of intestinal protozoans were defined as *Blastocystis spp*, 26.7% (8/30) as *C. parvum*, 20% (6/30) as *G. duodenalis* and 13.3% (4/30) as *E. histolytica* (Table 4).

In our study, the microscopic method was taken as the gold standard and the sensitivity and selectivity of the diagnostic methods used in the detection of intestinal protozoans in COVID-19 patients were compared. It was determined that the real-time PCR analyses, one of the diagnostic methods, was most sensitive than the other diagnostic methods, followed by PCR ELISA and microscopic analyses. It was found that real-time PCR was 99.1% sensitivity, 95.9% PCR sensitivity, 93.5% ELISA sensitivity and 91.3% microscopic method sensitivity in this study. Since the sensitivity is higher than other methods, the distribution of intestinal protozoans according to the symptoms of COVID-19 patients included in the study was evaluated according to the results of this real-time PCR method. In this study, 4.2% (2/48) *Blastocystis spp*. was detected in COVID-19 patients with mild clinical symptoms, no other intestinal protozoans was found in this patient group. *Blastocystis spp*. (7.5%, 3/40) and *G. duodenalis* (5%, 2/40) intestinal protozoans were detected in moderate clinical symptoms COVID-19 patients. However, it was detected 13.3% (8/60) *Blastocystis spp*., 15% (9/60) *C. parvum*, 8.4% (5/60) *G. duodenalis*, 6.7% (4/60) *E. histolytica* in severe clinical symptoms COVID-19 patients (Table 5). The values of CD4$^+$ T cell and CD8$^+$ T cell have been observed to every low in the patient group of severe clinical symptoms where intestinal protozoan was detected. It was observed that CD4$^+$ T cell and CD8$^+$ T cell values were low in COVID-19 patients where *C. parvum* were detected and COVID-19 infection recurred in these patients.

Table 5: Distribution of intestinal protozoans according to clinical symptoms of COVID-19 patients.

Protozoans species	Mild (n=48,%)	Moderate (n=48,%)	Severe (n=60,%)
Blastocystis spp.	4.2	7.5	13.3
C. parvum	0	0	15
G. duodenalis	0	5	8.4
E. histolytica	0	0	6.7

According to the results of DNA sequence analysis each clinical samples of *Blastocystis spp*., *C. parvum*, *G. duodenalis* and *E. histolytica* corresponded with the sequence of intestinal protozoans reported in GenBank. The 98-100% similarity was found between the nucleotide sequences of intestinal protozoans isolated from COVID-19 patients and the nucleotides of intestinal protozoans previously registered in GenBank. As shown in Figure 1, *Blasto-*

cystis spp., *C. parvum*, *G. duodenalis* and *E. hisolytica* were in different classes due to different nucleotide sequences in the phylogenetic tree, and *C. parvum variant 1* isolated from recurrent COVID-19 patients was in a different class. The genetic variant value was found between 0.05 (27/517) and 0.07 (37/310) among *C. parvum* isolates in the study (Figure 2). In the phylogenetic tree, *C. parvum* variants isolated from recurrent COVID-19 patients were in a different class from other *C. parvum* isolates. However, no mutation was found in the nucleotide sequences of other intestinal protozoans isolated from mild, moderate and severe clinical symptoms COVID-19 patients. Intestinal protozoans isolated in the study were registered in GenBank with numbers *Blastocystis spp.* OQ703028, *G. duodenalis* OQ703029, *C. parvum* OQ703032, *C. parvum variant 1* OQ703030, and *E. histolytica* with the number OQ703031, respectively.

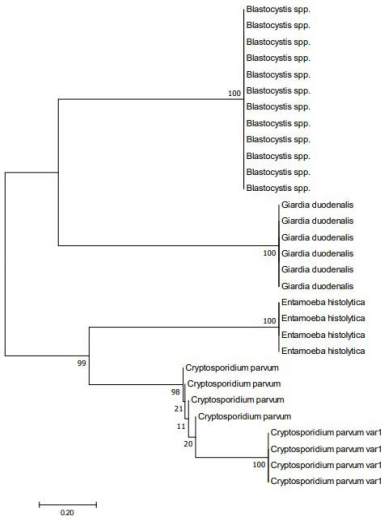

Figure 1: Phylogenetic tree appearance of intestinal protozoan isolated from COVID-19 patients. Phylogenetic tree of the different gene region nucleotide sequences of intestinal protozoans isolated from COVID-19 disease. The tree was constructed using Tamura 3-parameter model in MEGA software version 6.05. Bootstrap values shown are based on 100 replicas. Bar represents genetic distance as reflected by nucleotide divergence. Bootstrap analysis showed a high degree of variant between 0.05 and 0.07 of C. parvum isolates isolated from recurrent COVID-19 patients.

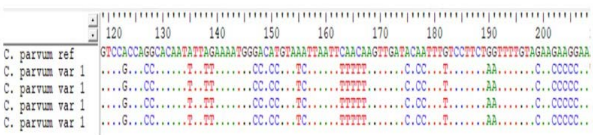

Figure 2: Variants in Cryptosporidium parvum nucleotide sequences isolated from recurrent COVID-19 patients. The results of DNA sequencing of C. parvum isolates and alignment of them. C. parvum (ref): ATCC PRA-67DQ™; C. parvum var 1 was isolated from recurrent COVID-19 patients. The most variable region consists of between 120 bp and 200 bp.

DISCUSSION

The COVID-19 disease, which is caused by the SARS-CoV-2 virus, was reported for the first time in the Wuhan province of China and has caused a worldwide pandemic for the last three years. Many studies have been conducted on the disease and one of these studies has shown that the severity of the disease is associated with the presence of comorbidities, such as age, diabetes, obesity and heart disease (Cao et al., 2020). In addittion, abnormalities in many laboratory parameters related to immune response such as neutrophil counts and proinfalammatory cytokines such as interleukin have been reported in severe COVID-19 patients (Kermali et al., 2020; Henry et al., 2019). Although studies on the pathogenesis of COVID-19 disease are still ongoing, the mechanism of the decrease in the number of lymphocyte of severe patients has not been fully elucidated. When the clinical symptoms of COVID-19 disease and the functions of $CD4^+$T cell, $CD8^+$T cell are evaluated together, it can provide prognostic information for the recovery process of the disease (Kermali et al., 2020; Henry et al., 2019).

The medical, public heath, and scientific communities have conducted various studies to contain COVID-19, develop effective vaccines, and identify complications of the disease (Aviv et al., 2020). The basis of these studies is the immune response against to the COVID-19 disease. Wang et al. (2020) have reported that, total lymphocytes, $CD4^+$T cell, $CD8^+$T cell, B cell, and NK cell decreased in COVID-19 patients, and these cell were at lower levels in severe clinical symptoms. In the study of Wang et al., especially $CD8^+$ T cell and $CD4^+/CD8^+$T cell ratio showed a significant relationship with inflammatory conditions in COVID-19 patients (Wang et al., 2020). The other studies on total lymphocytes in COVID-19 patients also reported that $CD4^+$T cell and $CD8^+$T cell were decreased in these patients (Li et al., 2020; Liu et al., 2020). Cellular immune function anda ge-related chronic diseases helpful determine the clinical progression of COVID-19 patients. In our study, it was observed that $CD4^+$T cell, $CD8^+$T cell values were low in severe clinical symptoms COVID-19 patients, and the number of these cell decreased with age. Our findings for $CD4^+$T and $CD8^+$T cell support previous study results.

It has been recognised that bacterial and fungal infections could also be seen in COVID-19 patients and it was added to the routine examinations, during the pandemic. How-

ever, it has been ignored that intestinal protozoans such as *Blastocystis spp.*, *C. parvum*, *G. duodenalis* and *E. histolytica* can be seen and these parasites can change the prognosis of COVID-19 disease. It is documented that parasitic infections such as ascariasis, trichuriasis alter patients' immune system towards type 1 immunity (IL-4, IL-5, IL-9 and IL-13). The result of this immune modulation is that people diagnosed with parasitic infections are at increased risk of contracting diseases such as HIV/AIDS, tuberculosis and malaria, due to the suppression of the basic imune response to intracellular pathogens (Gluchowska et al., 2021). Since there is a decrease in the cell that make up the immune response system in COVID-19 patients, these patients have a high risk of getting parasitic infections.

Abdoli has been reported that comorbidities of helminths such as *Ascaris spp.*, *Taenia spp.*, can suppress the effective immune response against SARS-CoV-2 virus and increase the morbidity and mortality of COVID-19 disease (Abdoli et al., 2020). Gluchowska et al. have been reported that the presence of parasites such as *Plasmodium spp.*, and *Schistosoma spp.*, in COVID-19 patients may increase the risk of death from COVID-19 disease (Gluchowska et al., 2021).

Abdel-Hamed et al. have classified COVID-19 disease according to the symptoms of patients and they have been reported parasitic infection in 78.2% of mild COVID-19 patients and 20.7% of severe patients (Abdel-Hamed et al., 2021). Several intestinal protozoans such as *Blastocystis spp.*, *C. parvum*, which were previously known to be non-pathogenic or have transient pathogenic potential in immnocompetent individuals, become opportunistic and aggressive in immunocompomised diseases such as in HIV/AIDS patients (Alemu et al., 2011).

Mouhand et al. have been reported that a diagonsis of asymptomatic filariasis in a COVID-19 patient who applied with complaints of shortness of breath, fever, and cough. The immunological interaction of both COVID-19 and filariasis is complex, but for the filariasis phenotype such as hydrocele, lymphedema, elephantiasis to occur, $CD4^+$T cell must be triggered (Mouhand et al., 2020). Sangare et al. have been reported that intestinal protozoans commonly cause widespread morbidity in HIV/AIDS patients due to the reduction of $CD4^+$T cell, and their incidence is high in countries where this type of disease is common (Sangare et al., 2015). Our study shows that intestinal protozoan disease are not only associated with symptomatic HIV-infected patients, but are also more prominent with reduced immune status, low $CD4^+$T cell count <200 cell/mm^3.

Some studies suggest that recovering from parasitic disease can strengthen the immune system and protect it from COVID-19 disease. Bamorovat et al. reported that those with a history of CL scarring significantly reduced the incidence of COVID-19 morbidity and mortality thanks to IL12 and IFNγ fundamental protection roles (Bamorovat et al., 2020). During the early phases of COVID-19 disease, appropriate local type-III and type-I IFN responses can eliminate SARS-CoV-2 virus or limit its replication, preventing disease progression to moderate and severe stages (Bamorovat et al., 2020). However, in the early stages of the disease, as a result of the patients also encountering parasitic infections, sufficient IFNs can not be produced and the COVID-19 disease may be exacerbated. Genetic variants in parasites may result in different phenoytpes that can be associated with the diversity of clinical manifestations and geographical distributions of diseases (Oryan et al., 2013). Huseein et al. have been stated that variants such as ACEI/D and ACE2 may be effective in COVID-19 patients with comorbidities parasite infection and may affect the prognosis of the disease (Huseein et al., 2020). The presence of variants in *C. parvum* isolates isolated from recurrent COVID-19 patients in our study indicates that genetic diversity in parasites affects the prognosis of the disease.

Intestinal protozoans and other parasites can influence the course of COVID-19 disease as risk factors or protective agents, therefore, the current coronovirus pandemic may affect the diagnosis and prevention and eradication programs of parasitic disease (Gluchowska et al., 2021). There are similarities and interactions between human parasitic diseases and clinical symptoms of COVID-19 disease. Cough, fever, shortness of breath and Löffer's syndrome are seen in patients due to parasites being trapped in the lungs or pulmonary capillaries during larval migration of parasites in the human body. These clinical symptoms are similar to the general symptoms of COVID-19 disease. Also, similar to the SARS-Co-V-2 virus, intestinal parasites can cause a variety of digestive symptoms such as nausea, vomiting, diarrhea and abdominal pain (Gluchowska et al., 2021). Due to the similarities between parasitic diseases and COVID-19 disease, the clinical symptoms of the two diseases of the patients should not be ignored in the clinical should be confirmed by laboratory method. The number of cases, geographical distribution and epidemiology of parasitic diseases differ from each other. However, the distribution of intestinal protozoans we detected in our study is cosmopolitan. For this reason, COVID-19 patients should be examined for these parasitic diseases in every region of the world, or COVID-19 disease shouldn't be ignored in those with these parasitic diseases.

It is known that COVID-19 patients with severe clinical symptoms have multiple comorbidities such as diabetes, hypertension, and cardiovascular diseases. As COVID-19 disease spreads and progresses, we will likely see many

comorbidities coming to clinician awareness. Our study shows that intestinal protozoan infections may also be comorbidities to COVID-19 disease. The comorbidities of intestinal protozoan infections in COVID-19 patients may be depressing in the treatment of COVID-19 disease and the development of an effective vaccine against this disease. The presence of variants in *C. parvum* isolates isolated from recurrent COVD-19 patients will be effective in the clinical investigation, epidemiology, prevention and control of the COVID-19 disease. For this reason, we recommend that further studies be conducted with more samples to investigate intestinal protozoans.

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CONFLICT OF INTEREST

None declared.

ETHICAL APPROVAL

The Ethical Committe of the Medicine Faculty of Necmettin Erbakan University gave approval for this study.

NOVELTY STATEMENT

It is known that COVID-19 shows more severe clinical symptoms in people with chronic diseases. However, the effect of intestinal parasites on the symptoms of the disease is unknown. The results of this study show thatintestinal protozoans affect the clinical symptoms of the disease. In particular, our study shows that C. parvum var-1 may be a causative agent in recurrent COVID-19 disease.

AUTHORS' CONTRIBUTIONS

FE conceived the study, analysed the data and wrote the sections on context; AA-CC-ST took stool and blood samples from COVID-19 patients and contributed to the writing of the paper.

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