Original Article | Iran J Med Microbiol. 2022; 16(5): 412-419

10.30699/ijmm.16.5.412

Iranian Journal of Medical Microbiology | ISSN:2345-4342

Effect of Cytokines Gene Expression and Serum Level of Vitamin D on the Severity of COVID-19

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ABSTRACT

Background and Aim: The COVID-19 disease is an emerging infectious disease that appeared in December 2019 in Wuhan, China. An uncontrolled systemic inflammatory response is one of the primary mechanisms causing death in this disease. In this study, the expression levels of some inflammatory cytokines, vitamin D, and some hematological and biochemical parameters were compared in patients with severe COVID-19 and mild types.

Materials and Methods: In this cross-sectional study, 60 blood samples were taken from 30 severe coronavirus patients and 30 mild coronavirus patients. The expression levels of cytokines such as IL (interleukin)-6, interferon (IFN)-α, IL-12, transforming growth factor (TGF) β, IL-8 and tumor necrosis factor (TNF)-α were evaluated using Real-time PCR. A T-test was used for Statistical Analysis.

Results: IL-6, IFN-α, IL-12, TGF-β, IL-8, and TNF-α cytokines in the peripheral blood of severe patients, were positive in 28/30 (93.33%), 27/30 (90%), 24/30 (80%), 25/30 (83.33%), 26/30 (86.66%), and 27/30 (90%) respectively. The positive rate of these cytokines in the mild patients were 20/30 (66.67%), 21/30 (70%), 18/30 (60%), 17/30 (56.67%), 19/30 (63.33%), 18/30 (60%), respectively. There was a statistically significant difference between these two groups in terms of cytokines biomarkers. A significant difference was found between both groups in terms of the serum level of lactate dehydrogenase (LDH), the mean number of lymphocytes and neutrophils as well as the mean percentage of neutrophils/ lymphocytes ratio (NLR).

Conclusion: The expression of cytokine genes and their release into the peripheral blood was increased in both severe and mild patients with COVID-19. However, they were more intense in patients with severe symptoms than those with mild symptoms and can cause inflammatory and even destructive reactions. Vitamin D deficiency plays no role in causing severe COVID-19 in patients without risk factors. Severe COVID-19 is characterized by elevated serum levels of LDH and NLR≥3.45.

Keywords: severe COVID-19, Mild COVID-19, ARDS, Cytokine expression

Received: 2022/07/01; **Accepted**: 2022/07/17; **Published Online:** 2022/08/08

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Kazempour Dizaji M, Jamaati H, bahrami N, Farzanegan B, Rekabi M, Mokhber Dezfuli M, et al et al . Effect of cytokines gene expression and serum level of vitamin D on the severity of COVID-19. Iran J Med Microbiol. Iran J Med Microbiol. 2022; 16 (5):412-9.

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1. Introduction

The current outbreak of coronavirus infectious disease 2019 (COVID-19) has recently spread to many countries throughout the world, which has caused a great deal of concern. Despite many efforts, currently, there is no vaccine or targeted treatment for COVID-19 **(1)**.

Most coronaviruses cause disease in certain species of hosts. Some cases can lead to a significant infectious threat to humans and cause severe respiratory disease **(2-4)**. Of course, Covid-19 is not the first respiratory disease caused by a coronavirus. However, in the last decade, there have been other epidemic diseases, such as Severe Acute Respiratory Syndrome (SARS) and the Middle East Respiratory Syndrome (MERS)**(5)**.

According to performed studies, the disease in people with Covid-19 may be severe or mild.

People with serious infections often have other underlying diseases, such as Diabetes, Immuno-deficiency, or cardiovascular diseases, which in turn accelerates the progression of the infection in them and sometimes leads to death **(6-9)**.

Patients with Covid-19 show clinical manifestations such as fever, cough, dyspnea, fatigue, and decreased Leukocyte count, and pneumonia radiography, which is actually similar to the symptoms of SARS-COV and MERS-COV **(6, 10)**.

According to a report in Lancet, ARDS is the main cause of mortality in patients with Covid-19. ARDS is a common immunological event for SARS-COV, 2-SARS-COV, and MERS-COV infections **(6)**.

One of the main mechanisms for ARDS is the Cytokine storm, which is, in fact, an uncontrolled systemic inflammatory response that causes death, which is due to release large amounts of pre-inflammatory cytokines such as IL-6, IL-12, IL -18, IFN-g, TNF-α and TGF-β **(11)**.

Accordingly, and considering clinical findings and manifestations, the number of inflammatory cytokines in people with severe Covid-19 infection will be different from the amount of these cytokines in patients with mild-moderate infection **(12)**.

Clinical diagnoses in infections such as Covid-19 can be done using biological samples such as blood and sputum through the Real-time PCR method, which also has good sensitivity and uniqueness **(13)**.

In this study, the expression evaluation of IL-6, IFN-α, IL-12, TGF-β, IL-8, and TNF-α cytokines in patients with severe Covid-19 and its comparison with mild type has been performed by Real-time PCR method.

2. Materials and Methods

Sample Collection

A total of 30 blood samples were taken from patients with severe COVID-19 who admitted to the ICU due to severe symptoms. Moreover, 30 people who the result of their CORONA test was positive, and had mild-moderate symptoms, and was hospitalized in the infectious-internal medicine wards participated in the study voluntarily. In both groups, patients were randomly selected, and patients who had similar symptoms but tested negative for Covid-19 were excluded from the study. The study was conducted as a case-control study in the period of 1400 in Masih Daneshvari Hospital, Iran.

Blood Preparation and RNA Extraction

After selecting subjects, 2 mL of peripheral blood was taken through a bleeding syringe in a tube containing the EDTA, and immediately the RNA extraction stage started.

RNA extraction was performed using the RNA Blood Mini Kit (Qiagen Cat no.52304) and according to Kit's protocol. RNA concentrations were determined using a spectrophotometer (NanoDrop, 2000, Thermo).

cDNA synthesis and Real-Time RT-PCR

RNAs were reverse-transcribed to cDNA using Viva 2-steps RT-PCR Kit cDNA Synthesis Kit (Vivantis, Malaysia). The expression levels of cytokines were determined using Cinna GreenqPCR Mix, 2X (Cina Colon, Iran) by real-time PCR (Applied Biosystems, USA). The reference gene 18srRNA was used as the normaliser in RT-qPCR. The sequences of the primer sets used are presented in the Table 1.

Real-time RT-PCR Reaction Components

The relative real-time PCR cycle consisted of initial denaturation at 95℃ for 5 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds, primers connection at 56°C for 1 minute, amplification at 72°C for 25 seconds and the final amplification at 72°C For 5 min. After completing each reaction, the interpretation of the results has been carried out based on the Amplification and Melting peak curves. To determine the gene expression amplification of patients with severe symptoms in patients with severe symptoms compared to patients with mild symptoms the $2^{-\Delta\Delta Ct}$ formula was used **(14-19)**. Three cDNA vials made from patients were tested to express the reference and the studied genes.

Table 1. Sequence and specifications of primers used

	IL-6	IFN-α	IL-12	TGF-β	IL-8	TNF-α	18srRNA
Forward primer	GGTACATCC TCGACGGC ATCT	GACTCCATCTT GGCTGTGA	TCAAACCAGA CCCACCGAA	CCCAGCATCTG CAAAGCTC	GAGAGTGATTGA GAGTGGACCAC	CCGAGGCAGTC AGATCATCTT	GTAACCCGTTG AACCCCATT
Length	21	19	19	19	23	21	20
Reverse primer	GTGCCTCTT TGCTGCTTT CAC	TGATTTCTGCT CTGACAACCT	GCTGACCTCC ACCTGCTGA	GTCAATGTACA GCTGCCGCA	CACAACCCTCTGC ACCCAGTTT	AGCTGCCCCTC AGCTTGA	CCATCCAATCG GTAGTAGCG
Length	21	21	19	20	22	18	20
product length	81	103	68	101	112	85	152
Annealing temperature	59	56	58	59	59	58	56

Statistical Analysis

The sample size was calculated according to the ratio of positive markers in both groups, and taking into account 5% and 20% of the first and second types of errors respectively.

The results were analyzed using SPSS Version 20, and the mean and standard deviations were also calculated. The paired t-test was used to analyze the difference or correlation between gene expression levels and Clinicopathologic Features. The difference was considered significant at P≤0.05.

3. Results

This study included 30 patients with COVID-19, who were admitted to the ICU due to severe symptoms, as well as 30 people who the result of their CORONA test was positive, and had mild-moderate symptoms, and were hospitalized in the infectious-internal medicine wards.

The group of patients with severe symptoms consisted of 21 males and 9 females, and the group of patients with mild symptoms consisted of 20 males and 10 females. There was no significant difference between two groups in terms of average age (Table. 2). Patients with severe and mild symptoms were tested for LDH and showed a significant increase in the mean serum levels of LDH in the ICU patients compared to the ward group (P= 0.003). The serum levels of CRP, vitamin D3, and hemoglobin showed no significant difference between two groups (Fig. 1 and Table 2). In the ICU patients the mean number of WBCs (P<0.001) and neutrophils (P= 0.004) were significantly increased compared to the ward group. However, the mean numbers of lymphocytes were significantly decreased in the ICU patients compared to the ward group (P= 0.001).

Table 2. Laboratory data diagrams information of patients with COVID-19 stratified by two clinical types

	group	N	Mean	Std. Deviation	P -value
Age	Ward	30	53.4667	16.48141	0.875
	ICU	30	54.2333	16.26225	
LDH	Ward	30	443.2593	201.78465	0.003
	ICU	30	969.3077	866.75716	
WBC	Ward	30	6.8817	2.42745	0.000
	ICU	30	11.3523	5.97583	
Hg	Ward	30	13.8833	1.69443	0.161
	ICU	30	13.1333	2.34864	
Neutrophil	Ward	30	64.8333	12.85753	0.004
	ICU	30	75.2667	14.36455	
Lymphocyte	Ward	30	25.4000	10.09814	0.001
	ICU	30	16.3000	10.43915	
CRP	Ward	30	0.3571	0.49725	0.120
	ICU	30	0.1176	0.33211	
VitD3	Ward	30	26.8333	15.05213	0.450
	ICU	30	41.8000	43.78584	

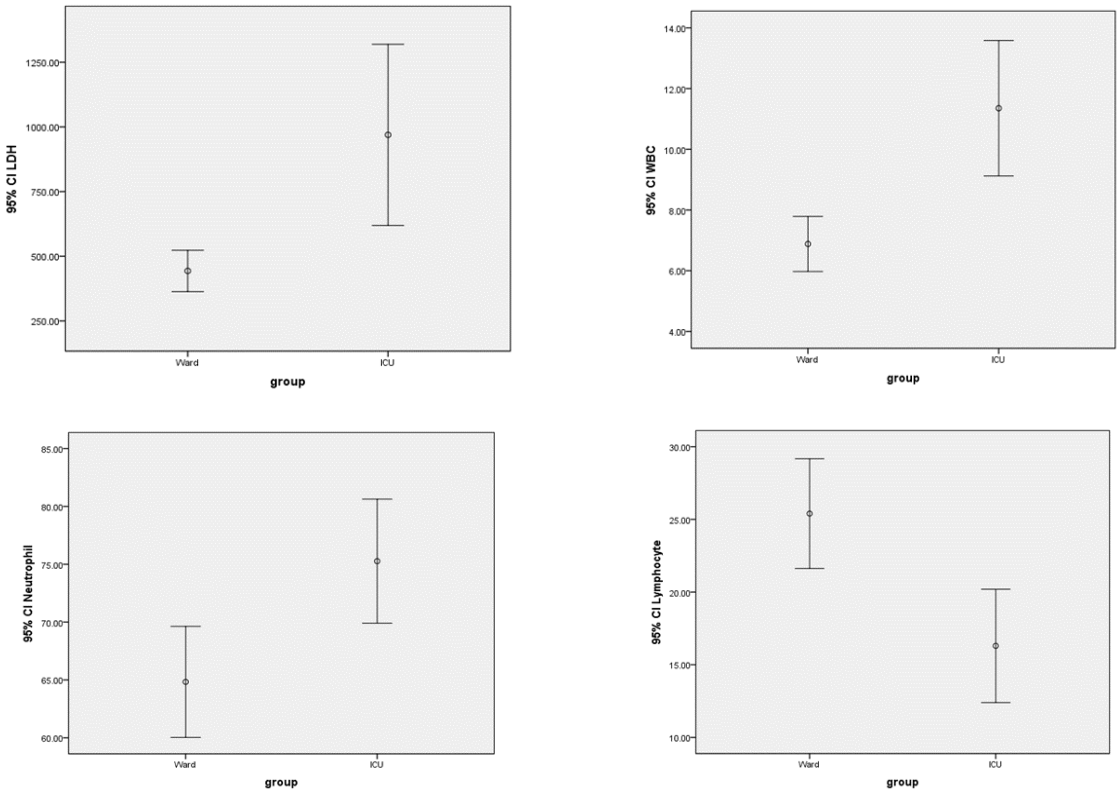

Figure 1. Laboratory data diagrams in severe and mild COVID-19 cases.

The Analysis of the Cytokines Expression

The relative expression of each gene varies in different patients. Evaluating the specific peak of the studied product can ensure that Real-time PCR is unique.

IL-6, IFN-α, IL-12, TGF-β, IL-8, and TNF-α markers in the peripheral blood of patients with severe symptoms, were positive on 28/30 (93.33%), 27/30 (90%), 24/30 (80%), 25/30 (83.33%), 26/30 (86.66%), and 27/30 (90%) respectively. The positive rate of these markers in the group of patients with mild symptoms was 20/30 (66.67%), 21/30 (70%), 18/30 (60%), 17/30 (56.67%), 19/30 (63.33%), 18/30 (60%), respectively. The statistical comparison of the positivity of these markers in both groups was performed using the two-sample binomial test, which showed that there was a statistically significant difference between these two groups (P< 0.001) (Fig. 2). The obtained data from gene expression levels showed that the relative expression level of IL-6, IFN-α, IL-12, TGF-β, IL-8, and TNF-α cytokines in the group of patients with severe symptoms versus the ward group were 1.08, 1.14, 1.09, 1.10, 1.22, and 1.12, respectively (Fig. 3).

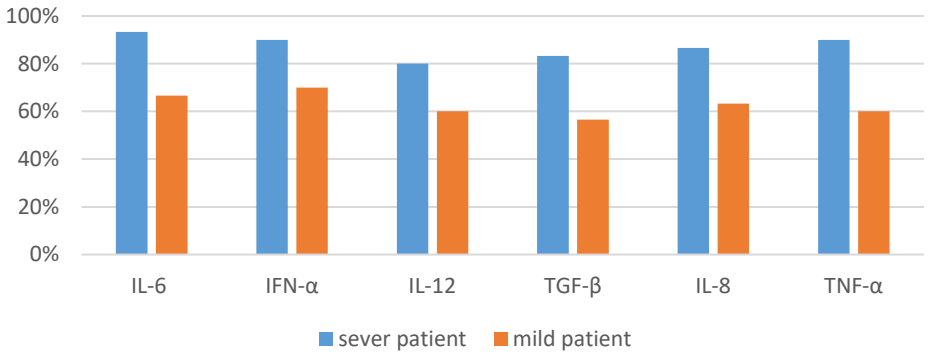

Figure 2. The rate of positive cytokines in patients with severe and mild symptoms.

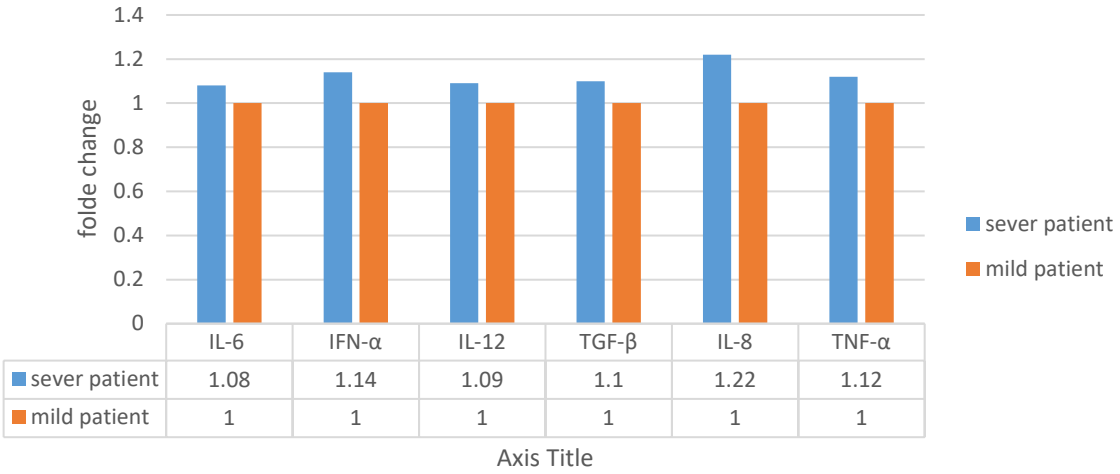

Figure 3. The difference between cytokine expressions in patients with severe symptoms compared to patients with mild symptoms.

4. Discussion

Cytokines are low molecular weight proteins or glycoproteins. These compounds can be considered as immune system hormones and inflammatory responses **(20, 21)**. Cytokines, in general, affect the activity, differentiation, amplification, and survival of immune cells, as well as regulate the production and activity of other cytokines, which can increase (pre-inflammatory cytokines) or decrease (anti-inflammatory cytokines) the inflammatory response **(21)**.

In the event of injury or infection in an organ or limb, an immune response is formed to suppress the infection, in which the release of pre-inflammatory cytokines during inflammation indicates the body's attempt to respond to the infection **(22)**. There is a dynamic and changeable balance in two directions, between pre-inflammatory cytokines and anti-inflammatory compounds of the immune system. Any change in this balance will lead to dangerous effects on the individual **(21, 23-25)**.

A number of cytokines play an important role in causing acute inflammation **(26)**. The development of the disease is strongly related to the expression level of inflammatory cytokines and chemokines **(27-30)**. Various cytokines and innate immune cells have been associated with immunopathology in coronavirus **(31-33)**.

This study examined the expression of several cytokine genes in patients with COVID-19 in a group with severe clinical symptoms and admitted to ICU, compared with the group with mild clinical symptoms hospitalized in the infectious-internal medicine wards and Real-time PCR technique is used in it. The technique has good sensitivity to molecular evaluations.

Several studies have shown that unregulated and excessive immune responses may lead to immunopathology and fatal disease in the patient **(34, 35)**. It is believed that host immune responses play a major role in the complications of diseases caused by SARS-CoV and MERS-CoV infections **(36)**.

In our study, using Ct values obtained from real-time PCR reactions and performed calculations, the increase of cytokine genes expression in patients admitted to the ICU with severe symptoms were shown compared with patients with mild symptoms admitted to infectious wards.

According to a report in Lancet, ARDS is the main cause of mortality in patients with Covid-19 **(37)**. Several pre-inflammatory cytokines such as IL-8 and IL-1β, are involved in the pathogenesis of ARDS **(38)**. ARDS is considered the main cause of mortality in patients with SARS-CoV or MERS-CoV **(38)**.

One of the main mechanisms for ARDS is the Cytokine storm, which is, in fact, an uncontrolled systemic inflammatory response that causes death, which is due to release large amounts of pre-inflammatory cytokines such as IFN-a, IFN-g, IL-1b, IL-6, IL- 12, IL-18, IL-33, TNF-a, TGFb, etc **(11, 39)**.

The study of C.K. Min *et al.* demonstrated that people with severe MERS-CoV infections, as those with SARS-CoV, reveal higher serum levels of IL-6, IFN-a, CCL5, CXCL8, and CXCL-10 compared to those with the mild disease **(12)**. In our study, an increase in the cytokines expression of IL-6, IFN-α, IL-12, TGF-β, IL-8, and TNF-α was observed in the peripheral blood of patients with severe symptoms compared with patients with mild symptoms.

In the study of Bandar Alosaimi *et al.* (2019), an increased inflammatory response was reported in patients with severe MERS-CoV infection that sometimes progressed to cause ARDS, which is in line with the present study, in which increased expression of inflammatory cytokines in patients with Severe COVID-19 was shown **(40)**.

The evidence of performed studies on SARS and respiratory syncytial virus (HRSV) has shown that there has been an increase in IL-8 with acute SARS infection, bronchiolitis, immunopathology, and disease enhancement during infection HRSV **(41)**. In a recent study, IL-8 also has shown an increase in severe cases.

5. Conclusion

In general, coronavirus infection, which affects human lungs, lead to inflammatory reactions in the human body, and the immune cells release a large amount of pre-inflammatory, inflammatory and anti-inflammatory cytokines that are called cytokine storms and these reactions cause serious complications and injuries in patients. It seems that in patients with more severe complications, who are admitted to the ICU, release of inflammatory cytokines, is more than patients with milder symptoms. The present study also showed an increase of IL-6, IFN-α, IL-12, TLF-β, IG-8 and TNF-α cytokine gene expression in the peripheral blood of patients with severe symptoms compared to patients with mild symptoms, which as a result of increasing the expression of these genes, the cytokines are produced and enter the bloodstream, causing inflammatory and even destructive reactions at their target sites.

It should be noted that the disease caused by the coronavirus (COVID-19), is a new disease, and in order to achieve more complete and comprehensive results, more research is needed for better diagnoses and treatments.

Ethics Approval

This study was approved by University ethical Committee (Ethical Code: IR.SBMU.NRITLD.REC. 1399.042) and all patients filled out an informed consent form.

Acknowledgment

This research is a part of the efforts of the professors and colleagues of Masih Daneshvari Hospital of Shahid Beheshti University of Medical Sciences. All involved are sincerely thanked.

Funding

None.

Conflict of Interest

There is no conflict of interest between the authors.

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