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MOLECULAR AND IMMUNE PROFILING OF INFLUENZA A/B INFECTION: ASSOCIATIONS BETWEEN VIRAL RNA BURDEN, ANTIVIRAL GENE EXPRESSION, AND SERUM CYTOKINES

Wisam H. Al-Shujairi *

Jabir Ibn Hayyan University for Medical and Pharmaceutical Sciences, College of Pharmacy, Iraq.

* Corresponding Author

ORCID Details

Wisam H. Al-Shujairi: <https://orcid.org/0000-0002-7727-3707>

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ABSTRACT

The aim of the current study was to define molecular and immune responses among patients with influenza infection and examine the effect of viral load and antiviral and inflammatory markers. Eighty participants were involved in the research: 40 influenza patients and 40 healthy individuals. Results showed that 67.4% of influenza A and 32.6% of influenza B were present with a mean Ct/Cq value equal to 25.8 ± 3.4 . Patients had higher average levels of the relative expression of IFNB1, MX1, and ISG15 genes (2.44 ± 1.05 , 3.44 ± 1.29 , and 3.61 ± 1.45 , respectively) relative to healthy controls (1.00), showing significant differences ($p < 0.001$). The levels of IL-6, TNF- α , IL-8/CXCL8, and IFN- γ were also higher in patients than in healthy controls ($p < 0.001$). In conducting the Spearman correlation analysis, it became evident that all markers studied show a positive correlation with viral load, as evidenced by the ρ values ranging between +0.626 and +0.757 ($p < 0.001$). The strongest correlation was observed with IL-8/CXCL8 and ISG15. The results indicate the joint activation of antiviral and inflammatory responses relating with viral load in patients with influenza.

Keywords: Influenza; Viral load; influenza A/B; IFNB1; ISG15; Cytokines

1 INTRODUCTION

Influenza infection represents a critical seasonal viral respiratory disease whose clinical effects are substantially due to a dynamic relationship between viral replication and the response of the immune system to the virus. Influenza A and British influenza B viruses are responsible for causing influenza virus infections in humans (Moriyama *et al.*, 2020). The presence of the virus and the type of virus causing the infection can be confirmed using molecular methods, particularly real-time reverse transcription PCR (RT-qPCR), which is a sensitive method of detecting the presence of viral RNA in respiratory samples (Peng *et al.*, 2024).

The viral RNA load is considered as an essential molecular factor in determining an infection, whereby it can be measured often using a calibrated RNA standard or can, in some instances, be qualitatively estimated including through the values of Ct/Cq in RT-qPCR conversions where lower values normally mean higher levels of the detectable viral RNA

and this is interpreted taking into account the specific characteristics and conditions of the measuring process (Bustin *et al.*, 2009; Saygan *et al.*, 2026).

In research carried out towards understanding influenza, one can see how scientists have used the method based on molecular measurement of avidity with the assessment of immune responses by other methods to study the relationship between viral load and the biological response of the organism (de Jong *et al.*, 2006; Almansa *et al.*, 2011).

The primary response of the innate immune system to influenza starts with the activation of type I interferon pathways. This is then followed by the stimulation of interferon-induced genes. The genes IFNB1, MX1, and ISG15 are crucial indicators of this process: IFNB1 begins the early interferon response; MX1 gene shows antiviral properties; and ISG15 gene is linked to the defense responses regulated by interferons and ISGylation. Laboratory studies have shown that influenza triggers the action of IFNB1, MX1, and ISG15, which are the components of innate antiviral response (Ramos *et al.*, 2019; Martin *et al.*, 2020).

Besides activating the antiviral response, influenza infection also initiates the activation of a wide range of inflammatory and immune mediators, including IL-6, TNF- α , IL-8/CXCL8, and IFN- γ . The levels of these cytokines are increased during natural or experimental influenza infection in humans, and certain cytokine response levels correlate with the level of viral replication or the clinical manifestation of the disease (Land, 2021).

The use of the ELISA method is significant in terms of measuring cytokine concentrations concerning the serum and plasma, as well as in determining the correlates of influenza infection (Bian *et al.*, 2014; Lee *et al.*, 2007). Thus, the connections among viral load, antiviral genetic response, and inflammation markers form the basis of a model that allows one to define the virus-host relationship. This connection can be analyzed statistically by Spearman's correlation coefficient in cases when data or relationships do not satisfy the requirements of parametric correlation (Almansa *et al.*, 2011; de Jong *et al.*, 2006).

Therefore, this study aimed to identify the molecular basis of Influenza A/B infection, measure the viral load, determine the expression of the genes IFNB1, MX1, and ISG15, measure the levels of IL-6, TNF- α , IL-6, and IFN- γ in blood and study how viral load correlates with the abovementioned markers

2 MATERIALS AND METHODS

2.1 Study Design and Participants

A case-control research was performed in Najaf Governorate, Iraq, with 80 persons enrolled in the study, consisting of 40 patients, who were confirmed to have Influenza A/B infection in the laboratory, and 40 healthy subjects. Cases were patients aged 18-65 years who had acute influenza disease within the last 72 hours. The diagnosis of influenza was mainly based on the use of real-time reverse-transcription quantitative PCR (RT-qPCR) for the confirmation of disease. Healthy controls were selected from the same community, based on the current age and gender. Participants receiving immunosuppressive treatment, individuals with immune defects, patients with active autoimmune disease, individuals with malignant tumors, pregnant women, and patients with other acute diseases were not included in the study. The study protocol was approved by the Institutional Ethics Board, and every subject involved in the study signed the informed consent.

2.2 Sampling of Clinical Data

A standardized questionnaire was employed to gather demographic and clinical data pertaining to age, gender, duration of symptoms, history of fever, presence of cough, sore throat, myalgia, and headache in subjects. Nasopharyngeal swabs were obtained utilizing sterile flocked swabs in 2–3 mL of viral transport medium, transported at a temperature of 2–8°C and stored at –70°C when separate processing was not carried out. Peripheral blood was obtained for serum separation via centrifugation at 1,500 g for 10 minutes and the recovered serum was frozen at –80°C until cytokine studies were conducted.

2.3 Extraction of Viral RNA and the Process of Influenza RT-qPCR

A total of 140 μ L of nasopharyngeal sample was taken for the extraction of viral RNA with the use of QIAamp Viral RNA Mini Kit (QIAGEN, Germany). It was done following the guidance of the manufacturer with use of the extraction negative control. The detection of Influenza A/B RNA was done with the help of a validated one-step RT-qPCR method based on the molecular detection guidelines of WHO with positive, negative extraction control and no-template control. In amplification, Applied Biosystems 7500 Real-Time PCR System or another method that was qualified was used. The positive samples were announced based upon the acceptance criteria for the assay and the values of Cq/Ct were kept in the records acting as the indicator of the viral RNA level.

2.4 RNA Extraction, cDNA Synthesis, and Gene Expression

To study gene expression in the host, total RNA was extracted from peripheral blood using a reliable RNA extraction system. Before reverse transcription, RNA's concentration and quality were measured. Synthesis of the cDNA was carried out using the SuperScript™ IV VIL0™ Master Mix (Invitrogen/Thermo Fisher Scientific) in accordance with the manufacturer's guidelines. The quantities of IFNB1 (Hs01077958_s1), MX1 (Hs00182073_m1), and ISG15 (Hs01921425_m1) were calculated using TaqMan real-time PCR, taking GAPDH (Hs02786624_g1) as the reference gene. The reactions were conducted by the TaqMan Universal Master Mix II with UNG on an Applied Biosystems 7500 platform. Each sample was tested in technical duplicates. The relative expression has been calculated by the formula $2^{-\Delta\Delta C_t}$, while the healthy control group was utilized for the calibration (Bustin et al, 2009).

2.5 Cytokine Analysis in Serum

The levels of serum IL-6, TNF- α , IL-8/CXCL8, and IFN- γ were measured with the use of sandwich ELISA kits obtained from companies producing kits for human use (R&D System/Bio-Techne, USA) as instructed by the kit Try. Both standard solution and samples were assessed in duplicate, and absorbance reading was made at the wavelength of 450 nm. The concentrations of cytokines can be expressed in terms of PG/mL.

2.6 Quality Control

In RT-qPCR assays, suitable positive, negative, and no-template controls were utilized along with duplicate technical reactions where amplification curves and Cq values were observed (Bustin et al., 2025). ELISA standards and samples were tested in duplicates, and test runs were accepted as per the quality requirements of the manufacturer (R&D Systems, Bio-Techne, 2025-2026).

2.7 Statistical Analysis

The analysis of the data was done using the IBM SPSS Statistics 29.0 software as well as the GraphPad Prism 10 software. The application of the Shapiro – Wilk test provided the distribution of the data. Normally distributed values were reported as mean $\pm$ SD and compared by the use of the independent-samples t-test. Non-normally distributed results were evaluated by the use of Mann – Whitney U test. The correlation analysis between the Ct/Cq index of viral RNA and some biomarkers was done using Spearman's rank correlation (ρ). The tests performed were bilateral with $p < 0.05$.

3 RESULTS

3.1 Demographic and Clinical Characteristics

The results of Table 1 showed a relatively similar mean age between patients with influenza (45.1 ± 8.4 years) and healthy individuals (42.4 ± 8.1 years), with a comparable gender distribution. Clinical symptoms among patients ranged from 60.0% for headache to 90.0% for cough, whereas these symptoms were not observed in the control group. The mean duration of symptoms was 2.8 ± 1.2 days.

Table 1: Demographic and clinical characteristics of influenza-infected patients and healthy controls

Variable	Influenza patients (n=40)	Healthy controls (n=40)
Age, years	45.1 ± 8.4	42.4 ± 8.1
Male, n (%)	24 (60.0)	22 (55.0)
Female, n (%)	16 (40.0)	18 (45.0)
Fever, n (%)	34 (85.0)	0 (0.0)
Cough, n (%)	36 (90.0)	0 (0.0)
Sore throat, n (%)	26 (65.0)	0 (0.0)
Myalgia, n (%)	28 (70.0)	0 (0.0)
Headache, n (%)	24 (60.0)	0 (0.0)
Fatigue, n (%)	30 (75.0)	0 (0.0)
Symptom duration, days	2.8 ± 1.2	—

Data are presented as mean $\pm$ SD or n (%).

3.2 Influenza A/B Detection and RT-qPCR Ct/Cq values

Influenza A virus was detected in 27 of the 40 infected patients (67.4%), while influenza B virus was detected in 13 patients (32.6%). The mean Ct/Cq value was 24.9 ± 3.3 for influenza A virus and 27.4 ± 3.1 for influenza B virus, with an overall mean of 25.8 ± 3.4 across all infected patients (Table 2).

Table 2: Influenza A/B RT-qPCR detection and Ct/Cq values among infected patients

Parameter	Influenza A (n=27)	Influenza B (n=13)	Total (n=40)
Positive RT-qPCR, n (%)	27 (67.4)	13 (32.6)	40 (100)
Ct/Cq value	24.9 ± 3.3	27.4 ± 3.1	25.8 ± 3.4

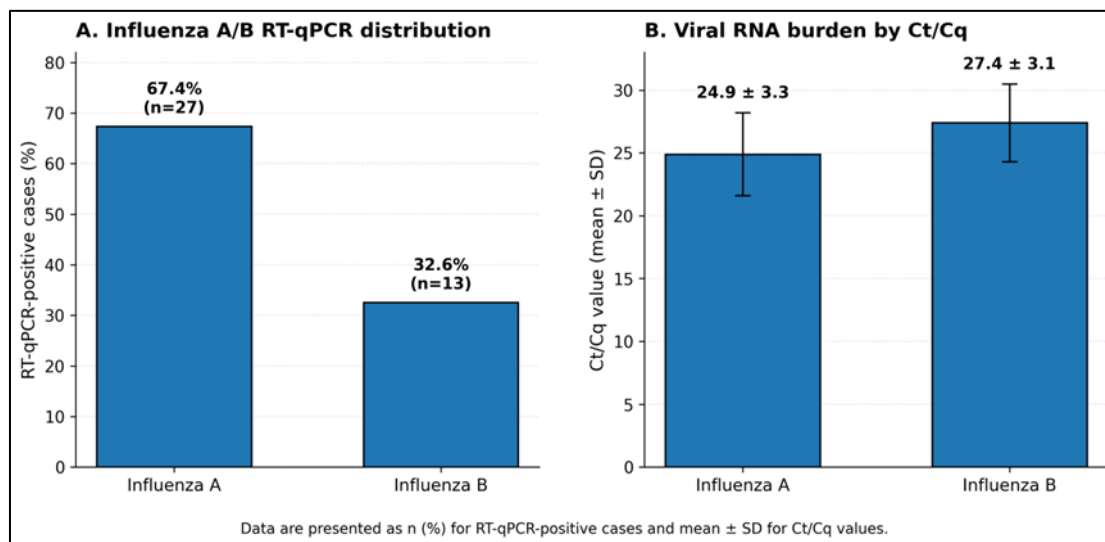


Figure 1: The distribution of the Influenza A and B viruses detected in patients using RT-PCR. (A) numbers of cases that were RT-qPCR positive for influenza A and B viruses; (B) mean Ct and Cq values of both viruses.

3.3 Expression of Antiviral Response Genes

The data of Table 3 indicated an increase in the relative expression levels of the IFNB1, MX1, and ISG15 genes in influenza patients compared to the control group; the values were 2.44 ± 1.05 , 3.44 ± 1.29 , and 3.61 ± 1.45 , respectively, versus 1.00 ± 0.22 , 1.00 ± 0.19 , and 1.00 ± 0.23 in healthy individuals, and all comparisons were statistically significant ($p < 0.001$).

Table 3: The relative expression of IFNB1, MX1, and ISG15 in patients infected with influenza and healthy subjects

Gene	Influenza patients (n=40)	Healthy controls (n=40)	p-value
IFNB1	2.44 ± 1.05	1.00 ± 0.22	<0.001
MX1	3.44 ± 1.29	1.00 ± 0.19	<0.001
ISG15	3.61 ± 1.45	1.00 ± 0.23	<0.001

Values are presented as mean ± SD of relative expression

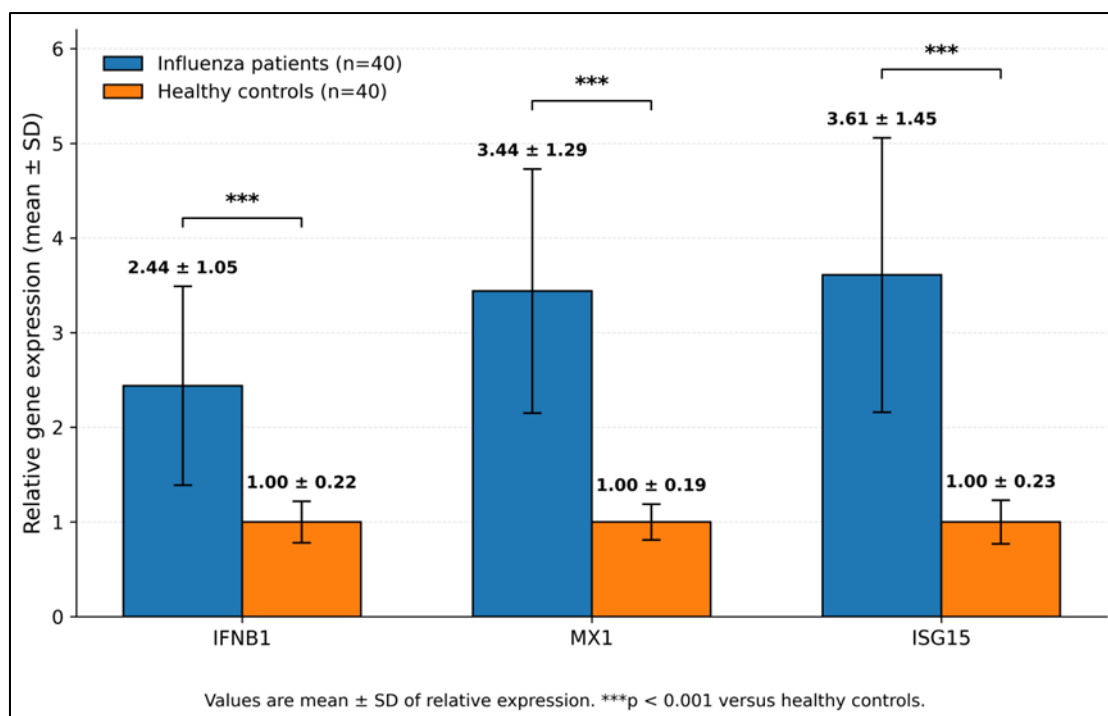


Figure 2: Relative expression of antiviral genes in those suffering from influenza and normal individuals. Relative expression level of IFNB1, MX1, and ISG15 evaluated using RT-qPCR and calculated using the $2^{-\Delta\Delta Ct}$ formula

3.4 Serum Inflammatory and Immune Biomarkers

The results of Table 4 showed elevated levels of all measured markers in influenza patients compared to healthy individuals. Specifically, the levels of IL-6, TNF- α , IL-8/CXCL8, and IFN- γ in patients were 36.6 ± 8.4 , 29.2 ± 6.4 , 46.5 ± 11.1 , and 22.3 ± 6.8 pg/mL, respectively, compared to 11.9 ± 3.5 , 8.8 ± 2.6 , 16.3 ± 4.2 , and 7.5 ± 2.3 pg/mL in healthy individuals (Figure 3). All differences were statistically significant ($p < 0.001$).

Table 4: Serum of inflammatory and immunity biomarkers in patients infected with influenza and in healthy participants

Biomarker (pg/mL)	Influenza patients (n=40)	Healthy controls (n=40)	p-value
IL-6	36.6 ± 8.4	11.9 ± 3.5	<0.001
TNF- α	29.2 ± 6.4	8.8 ± 2.6	<0.001
IL-8/CXCL8	46.5 ± 11.1	16.3 ± 4.2	<0.001
IFN- γ	22.3 ± 6.8	7.5 ± 2.3	<0.001

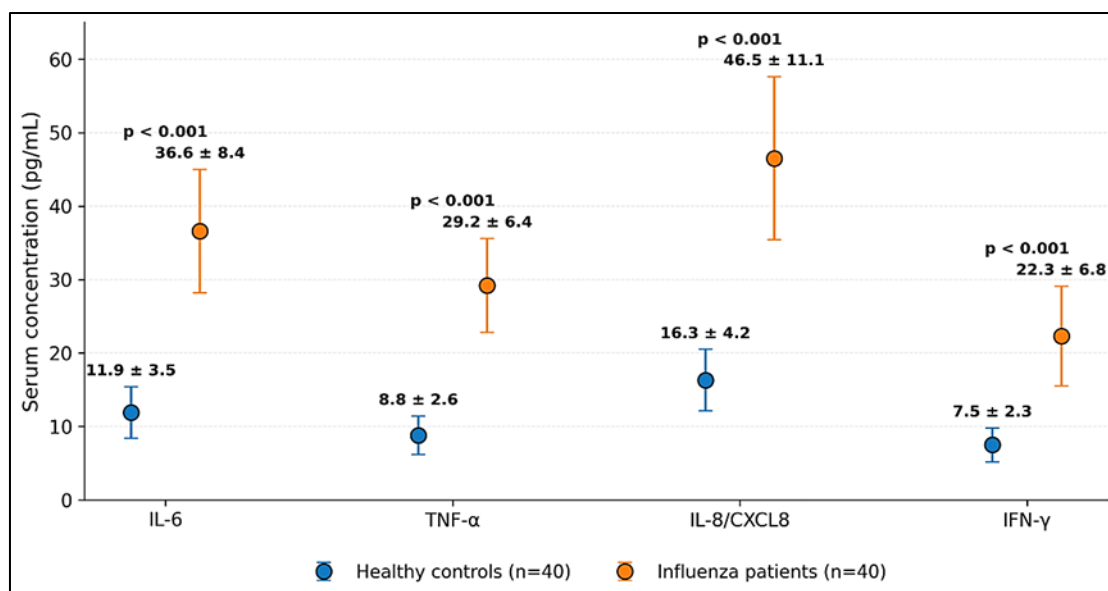


Figure 3: Serum inflammatory and immune biomarkers concentration found in patients with influenza and individuals that do not have the disease. Serum levels of IL-6, TNF-α, IL-8/CXCL8, and IFN-γ measured using ELISA

3.5 The correlation of levels of RT-qPCR Ct/Cq with those of host biomarkers

As shown of Table 5, significant positive correlations were observed between RT-qPCR Ct/Cq values and the measured antiviral and inflammatory biomarkers, with Spearman's ρ values ranging from +0.626 to +0.757 ($p < 0.001$). The strongest correlations were observed for IL-8/CXCL8 ($\rho = +0.757$), ISG15 ($\rho = +0.756$), and MX1 ($\rho = +0.746$), followed by TNF-α ($\rho = +0.738$), IFNB1 ($\rho = +0.704$), IFN-γ ($\rho = +0.693$), and IL-6 ($\rho = +0.626$).

Table 5: Spearman correlations between the RT-qPCR Ct/Cq values and antiviral/inflammatory biomarkers of patients suffering from influenza

Variable	Spearman's ρ	p-value
IFNB1	+0.704	<0.001
MX1	+0.746	<0.001
ISG15	+0.756	<0.001
IL-6	+0.626	<0.001
TNF-α	+0.738	<0.001
IL-8/CXCL8	+0.757	<0.001
IFN-γ	+0.693	<0.001

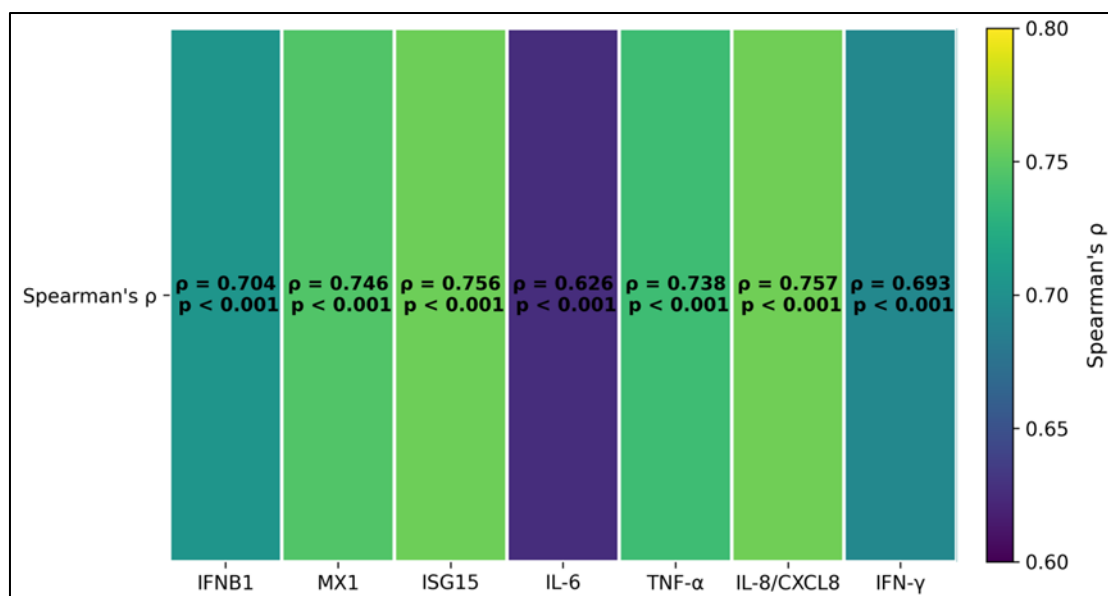


Figure 4: The relation between results of RT-qPCR and level of antiviral and inflammatory biomarkers present in patients suffering from influenza. Correlation coefficients for the level of IFNB1, MX1, ISG15, IL-6, TNF- α , IL-8/CXCL8, and IFN- γ are given

4 DISCUSSION

Based on the study, cough, fever, fatigue, and myalgia were found to be the clinical symptoms commonly associated with acute influenza infection, without any considerable disparities in age and sex distribution between the two groups. Similarly, this is consistent with other clinical studies that have shown how acute influenza infection is linked with the occurrence of inflammatory responses and development of systemic symptoms (Hayden *et al.*, 1998; Kaiser *et al.*, 2001).

Among the two types, Influenza A had a higher prevalence than Influenza B with mean Ct/Cq values indicating variation between them. The presence of both subtypes calls for the significance of molecular characterization of seasonal strains of virus whereby it has been proven by clinical studies that there is a difference in cytokine response patterns of Influenza A and B infections (Wang *et al.*, 2015).

There was a considerable rise in the relative expression of three genes, IFNB1, MX1, and ISG15, in patients when compared to that of healthy individuals. This indicates the activation of the interferon pathway and the antiviral gene response IFNB1 mediates the early response to infection, MX1 acts as an anti-influenza effector, and ISG15 is an interferon-induced gene that is involved in antiviral defense (Ronni *et al.*, 1997; Perng & Lenschow, 2018).

Compared to healthy individuals, the levels of IL-6, TNF- α , IL-8/CXCL8, and IFN- γ were significantly elevated in patients. This confirms that there has been activation of inflammatory response, as well as enhanced cytokine response in cases of influenza infection. There are studies that correlate levels of IL-6, TNF- α , and other cytokines with viral activity and clinical manifestations of the illness in humans (Kaiser *et al.*, 2001; Hayden *et al.*, 1998).

One finding and most important was that there are positive statistically significant correlations between the viral load and all investigated markers. The best correlations were detected with IL-8/CXCL8, ISG15, and MX1. This indicates that the results are in line with previous research proving the relationship between the viral activity and increased cytokines and inflammatory response, therefore supporting the link between increased viral activity and activation of the interferon pathway and interferon-stimulated genes (Hagau *et al.*, 2009; Perng & Lenschow, 2018).

The data overall suggest that influenza virus infection involved the simultaneous activation of both antiviral and inflammatory gene responses coupled with the presence of a connection between the viral load and the host markers. These results support the idea that the immune response to influenza signifies a phenomenon where viral replication interacts dynamically with innate and inflammatory defense systems (Iwasaki & Pillai, 2014).

5 CONCLUSION

According to the research findings, the viral infection is linked with the ongoing activation of both innate immunity and inflammatory responses, as manifested by a noticeable rise in the expression of the genes IFNB1, MX1 and ISG15, along

with the corresponding elevation of the cytokines, such as IL-6, TNF- α , IL-8/CXCL8 and IFN- γ compared to normal controls. The findings also indicate that there are significant positive correlations between viral load and the different immunological and genetic markers such as ISG15, IL-8/CXCL8, and MX1, which suggests an interrelationship between viral activity and regulatory mechanisms involved in the antiviral and inflammatory processes. As a result, the objectives of the present study are to conduct molecular characterization of Influenza A/B, to evaluate viral load, to quantify expression of IFNB1, MX1, and ISG15 genes, to measure serum levels of IL-6, TNF- α , IL-8/CXCL8, and IFN- γ , and to examine association between viral load and these markers.

STATEMENTS ON COMPLIANCE WITH ETHICAL STANDARDS

Disclosure of conflict of interest

No conflict of interest to be disclosed.

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