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Tumor necrosis factor Alpha gene polymorphism in patients with Influenza A pdm/09 in Nepal

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ABSTRACT

More than millions of people are living under the threat of influenza virus over the world countries including Nepal. The pathogenesis of this disease is not clearly understood and is probably attributed to genomic variations in viral strains as well as the host genetic makeup. The present study is to determine the role of polymorphism of TNF-alpha promoter regions at positions -238G/A, -308G/A, -857C/T and -863C/A in the severity of Influenza A Pdm/09 patients. Total of 500 patients including 300 Influenza A Pdm/09 (RT-PCR positive) and 200 apparently healthy individuals (Influenza A Pdm/09 Negative by RT-PCR) were included in the study. Polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) using site specific restriction enzymes were implemented for polymorphism study of TNF alpha promoter. Following the analysis of the digestion patterns of four polymorphic sites of the TNF- alpha promoter region, a significant association was observed between the allele -308A with the patients of Influenza A Pdm /09 patients. TNF- alpha 308 G/A has been shown to be associated with elevated TNF- alpha transcriptional activity. As per the literature search, this is the first study to identify the role of TNF- alpha promoter in Influenza A Pdm/09 infection. Our results show that subjects with - 308A more vulnerable to the severe form of Influenza A Pdm/09 infection

Introduction

In early April, a new Influenza A (H1N1) virus emerged among human since California and Mexico, quickly spreading worldwide through human-to-human transmission, and generating the first influenza pandemic of the 21 century. The virus was reported to be antigenically unrelated to the seasonal influenza viruses but genetically related viruses found to circulate among pigs. Because of its most likely swine origin, it is often called as 'Swine origin influenza virus'(S-OIV) A/H1N1, or pandemic influenza A (H1N1) 2009 virus [1]. Molecular analysis of the new Influenza A (H1N1) 2009 pandemic virus genome showed that it was derived from several viruses which have been circulating in pigs for years, namely the North American H3N2 triple-reassortment, the classical swine H1N1 lineage, and the Eurasian 'avian-like' swine H1N1 virus [2].

The infection was rather self limiting, with symptoms of illness of the upper respiratory tract. The ubiquitous characteristic of this H1N1 pandemic compared with other influenza A pandemics was the occurrence of more severe cases among young individuals. Severity was defined by thead vent of pulmonary complications, namely pneumonia, acute lung injury (ALI), and adult respiratory distress syndrome (ARDS). These were also involved in pregnant women and among individuals with underlying diseases such as obesity, bronchial asthma, and heart failure [3].

Viral infections are in general self-contained after activation of the innate immune defense. Viral particles are recognized by pattern recognition receptors of the innate immune cells, mainly blood monocytes and tissue macrophages, leading to the release of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin (IL)-1b, IL-6, and IL-8 [3]. The H1N1 virus causes more severe pathological lesions in the lungs of infected mice, ferrets and non-human primates than seasonal human Influenza virus. The host immune responses have been implicated in contributing to severe respiratory pathogenesis of nvH1N1 infections. Even in study conducted by Bermejo *et al*. has shown that Th1 responses (IFNγ, TNF-α, IL-15) were elicited in severe patients infected by H1N1 patients rather than controls [4].

Tumor necrosis factor- α is a pro-inflammatory cytokine [5,6]. Although the controlled self–limited expression of TNF plays a critical role in activating host defense mechanisms but uncontrolled over expression of TNF accounts for the devastating consequences for the host, consequently leading to diffuse inflammation, multi-organ dysfunction, hemodynamic collapse and cardiomyopathy [7]. Recent studies have found that levels of circulating TNF-α is elevated in viral Hepatitis, Japanese encephalitis, avian influenza postulating its contribution to the progression of the

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disease process. Polymorphism in the promoter region of the TNF-α gene might link with genetic basis of the diseases. The reasoning behind the proposed involvement of TNF gene polymorphisms in diseases is based on the production of various cytokines *in vivo* following the infection and the observation of single nucleotide polymorphisms (SNPs) within the gene segment.

Given the biological regulation of TNF-α and its role in the inflammatory process, it is perhaps surprising that the genetic influences on cytokine production have much influence on disease process and their outcome. Under circumstances where the release of TNF-α has been triggered the genetically endowed capacity for greater TNF-α production leads to severe inflammatory reactions. Till date 621 SNPs in TNF-α gene have been reported in the NCBI website, with more than 10 SNPs in the promoter region, including -238 G/A , -244 A/G, -308 G/A, -376 A/G, -575A/G,-857C/T,-863C/A,-1031T/C [8,9]. Regarding the TNF gene, four SNPs have been well associated with the higher TNF-α production after viral infections [10,11], all at different locations within the promoter region : a substitution of guanine by adenine at the -238 position (-238 TNF G/A), a substitution of guanine by adenine at the -308 position (-308 TNF G/A), a substitution of cytosine by thymine at the -857 position (-857 TNF C/T), a substitution of cytosine by adenine at the -863 position (-863 TNFC/A).

Influenza viruses associated with seasonal influenza cause tissue damage mainly through virus induced cell death. On the other hand, H5N1 infections in humans were associated with massive cytokine storm. It is found that the swine flu H1N1 virus causes more severe pathological lesions in the lungs of infected mice, ferrets and non-human primates than the seasonal influenza virus [12]. The present study is proposed to look into the SNPs in the TNF-α promoter region in laboratory confirmed Pandemic H1N1 patients

Methods

Study Design and Sample Collection

All the subjects (n:500) included in the study were enrolled during the post monsoon period (2017-2019) i.e. influenza transmission season with informed consent. Altogether 300 samples were collected either at NPHL or at sentinel sites of National Influenza Surveillance Network using swabs with synthetic tips. The samples collected from sentinel sites were transported to NPHL at 2 – 4^{o}C within 24 hours which were then stored at -70^{o}C till further processing and the rest (n: 200) Two hundred age and sex matched asymptomatic healthy individuals were included as control. Patients included in the study had either influenza like illness (ILI) that includes fever, cough, running nose, chills, sore-throat or, severe acute respiratory infection (SARI) or pneumonia which were in accordance with WHO case definition for ILI and SARI.

Sample Processing

All the samples in viral transport media were allocated into two microcentrifuge tube following collection among which one was used for RNA extraction to be used for PCR and the other was stored at -70^{o}C. RNA of influenza viruses were extracted using QIAamp® Viral RNA Mini Kit (QIAGEN GmbH, Hilden, Germany) following manufacturer's recommended procedure which was then used for nucleic acid detection.

Nucleic acid amplification was done using real time PCR thermal cycler (Rotor-Gene 6000 Corbett. Australia) which include three major steps; 1) reverse transcription at 95°C for 30 minutes, Taq inhibitor activation at 95°C for 2 minutes and 3) PCR amplification for 45 cycles at 95°C for 15 seconds, followed by 55°C for 30seconds.

Work plan of study are shown in Figure 1

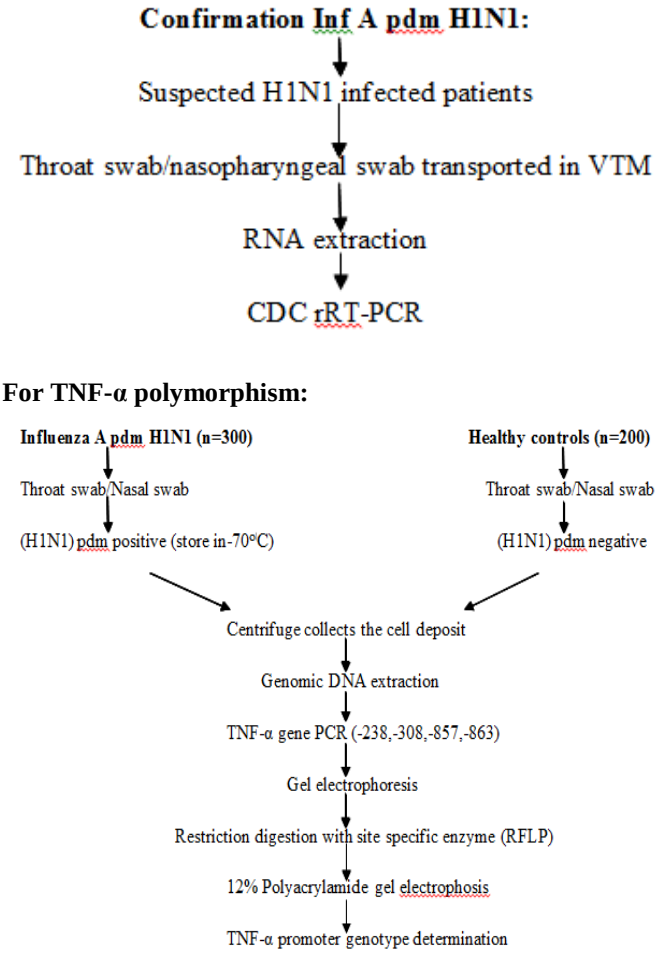

Figure.1 Flowchart of work design

The primer and probes used in the reaction mixture for identification of different types and subtypes of influenza virus are shown table (1,2)

Table 1. Nucleic acid amplification condition

Reverse Transcription	50°C for 30 min
Taq inhibitor activation	95°C for 2 min
PCR amplification (45 cycles)	95°C for 15 sec 55°C for 30 sec*

Table 2. Primers used

Positions	Primer sequences(5' to 3')	PCR product (bp)
-238 G/A	F: AGAAGACCCCCCTCGGAACC R: ATCTGGAGGAAGCGGTAGTG	152
-308 G/A	F: AGGCAATAGGTTTTGAGGGCCATG* R: ACACACAAGCATCAAGGATAC	143
-857 C/T	F:AAGTCGAGTATGGGGACCCCCCGTTAA* R: CCCCAGTGTGTGGCCATATCTTCTT	131
-863 C/A	F:ATGTAGCGGCTCTGAGGAATGGGTTACA R: CTACATGGCCCTGTCTTCGCCAAG*	133

Data analysis

The collected data was entered into and analyzed using Statistical Package for Social Sciences (SPSS) software version 20.0 [IBM Armonk, NY, USA].

Ethical Consideration

Ethical approval was obtained from National Public Health Laboratory and Nepal Health Research Council (NHRC ref. no. 1673) before carrying out this study.

Results

Demographic characteristics of patients

The present study included 500 throat swab samples from which 300 confirmed Influenza A pandemic H1N1 cases were found and 200 patients were apparently healthy individual. The age of both cases and controls were in the range of 1-65 years. The samples were subjected for TNF-α polymorphism detection by PCR – RFLP method. 300 conform influenza A/Pdm09 is shown in Figure 2.

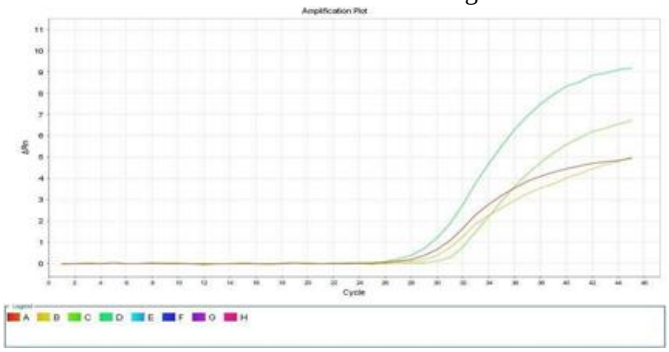

Figure.2 The amplification shows the virus positivity of Influenza A/H1N1

TNF-α gene promoter PCR

DNA was extracted from all test and control groups. The DNA of samples were subjected to PCR of TNF-α gene promoter regions -238G/A, -308 G/A, -857 C/T and -863 C/T. The amplified product size obtained 152bp, 143bp, 131bp, and 133bp respectively in figure3, 4, 5 and 6.

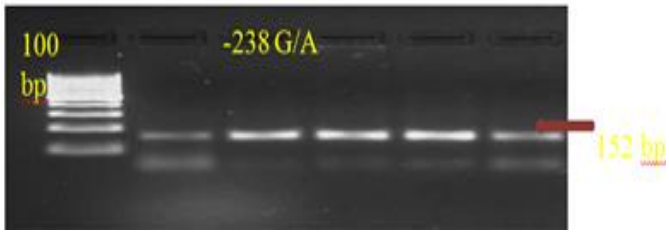

Figure. 3 TNF-α gene -238 region showing band size at 152bp

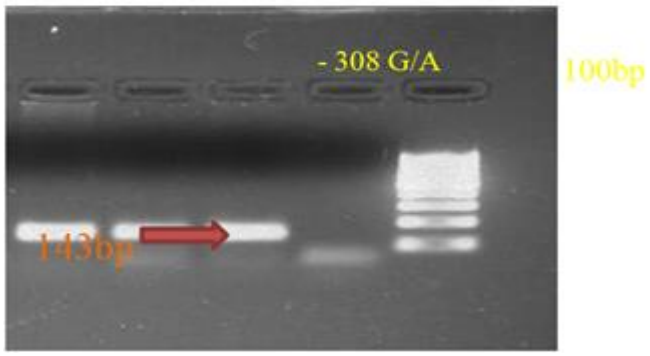

Figure. 4 TNF-α gene -308 region showing band size at 143bp.

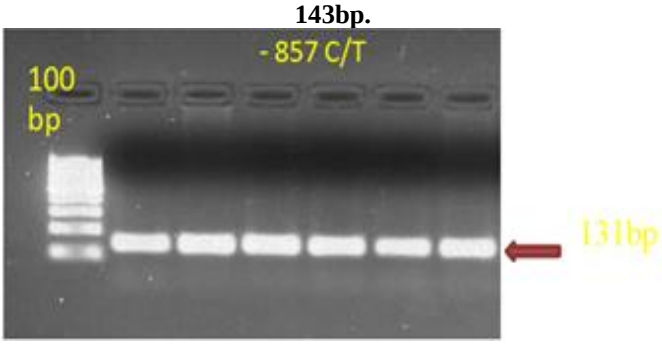

Figure.5 TNF-α gene -857 region showing band size at 131bp

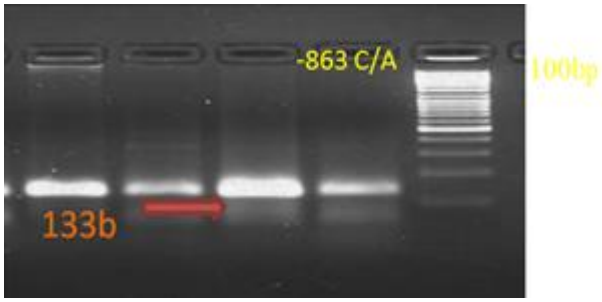

Figure.6 TNF-α gene -863 region showing band size at 133bp

Restriction Fragment Length Polymorphism (RFLP)

These PCR products were further subjected to restriction digestion with respective restriction enzymes as shown below and the digested products were observed in ethidium bromide stained 3% agarose gel. The allelic type was determined according to the presence or absence of the digested product of the desired length Table 3.

Table: 3 Restriction enzymes and digestion patterns for genotyping of TNF-α promoter region.

Positions	Restriction enzyme	Digested products	Genotype
-238 G/A	*Msp* I	132,20	GG
		152,132,20	GA
		152	AA
-308 G/A	*Sty* I	123,20	GG
		143,123,20	GA
		143	AA
-857 C/T	*Hinc* II	106,25	CC
		131,106,25	CT
		131	TT
-863 C/A	*Sty* I	108,25	CC
		133,108,25	CA
		133	AA

Restriction enzymes and digestion patterns for genotyping four different type of TNF- Alpha promotor region are shown in Figure (7, 8, 9 and 10)

-238 G/A with Msp I

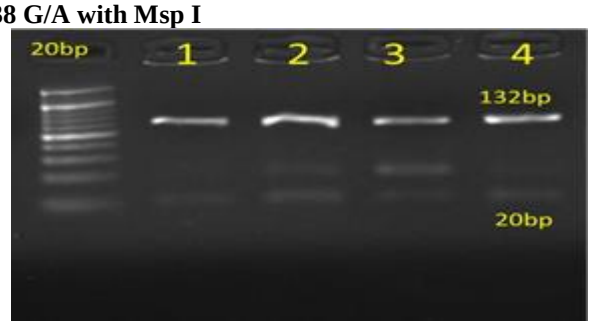

Figure 7. Visualization of the PCR digested products of -238 region

Lane 1st molecular size marker, Lane 1:20 and132 bp, Lane 2: 20 and 132 bp and Lane 3: 20 and 132 bp.

308 G/A with styI

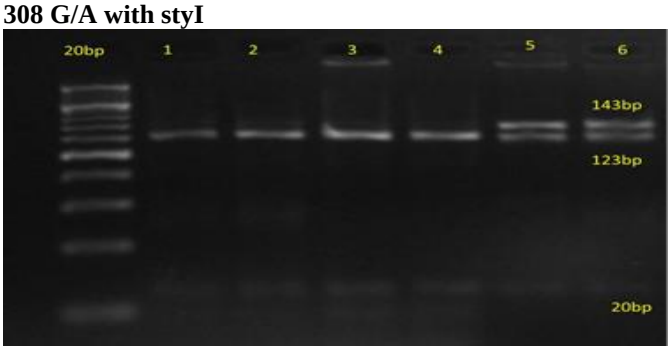

Figure 8. Visualization of the PCR digested products of -308 region

Lane 1st: Molicular marker, Lane 1-4: 123 bp and Lane 5-6: 143, 123, 20 bp.

-857 C/T with Hinc II

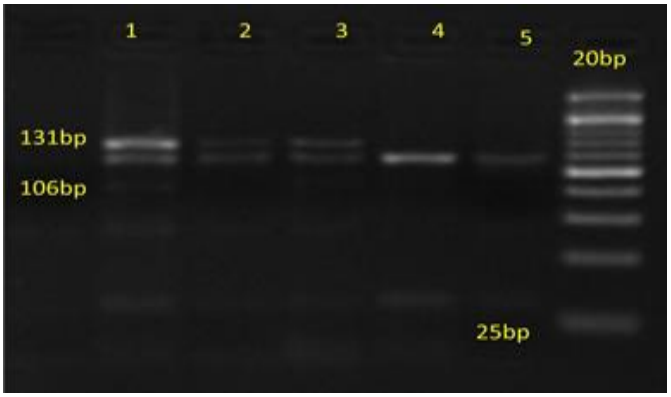

Figure 9. Visualization of the PCR digested products of -857 promoter region

Lane last: Molecular marker, Lane 1-3: 25, 106 and 131 bp, Lane 4: 131 and 25 bp and Lane 5: 131 and 25 bp.

-863 C/A Sty I

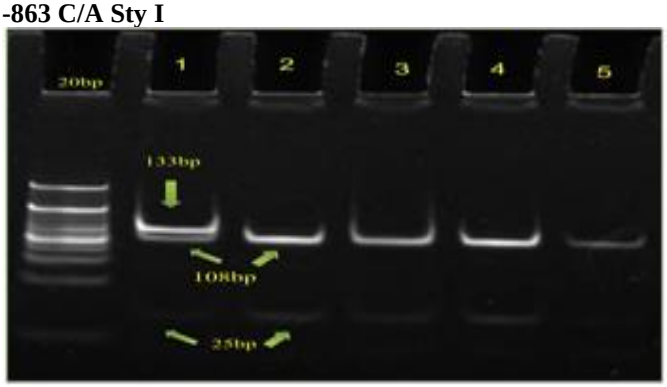

Figure. 10 Visualization of the PCR digested products of -863 promoter region.

Lane 1st: Molecular marker, Lane 1: 133,108 and 25 bp, Lane 2: 108 and 25bp, Lane 3: 108 and 25bp, Lane 4: 108 and 25bp and Lane 5: 108 and 25bp

Genotypic distribution and allelic frequency

The genotypic and allele frequency distribution for the four polymorphisms of -238G/A, -308 G/A, -857 C/T and -863C/A shown in table below (Table 4).

Table.4 Genotypic and allelic frequencies of TNF-α polymorphism

Loci	Genotype	Cases (N=300)	Control (N=200)
238	GG	(270/300)90%	(200/200)100%
	GA	(20/300)6.6%	(0/200)0
	AA	(10/300)3.3%	(0/200)0
308	GG	(81/300)27%	(170/200)85%
	GA	(219/30)73%	(30/200)15%
	AA	(6/30)20%	(0/200)0
863	CC	(190/300)63.3%	(90/200)45%
	CA	(80/300)26.6%	(90/200)45%
	AA	(20/300)6.6%	(20/200)10%
857	CC	(240/300)80%	(140/200)70%
	CT	(40/300)13.3%	(50/200)25%
	TT	(20/300)6.6%	(10/200)5%

Statistical difference was observed in the genotypic and allelic frequency distribution of -308 TNF-α promoter loci between the cases and controls as shown in Table 5.

Table. 5 Statistical comparison of Genotypic and allelic frequencies of TNF-α polymorphism between cases and control groups

Loci	Genotype	H1N1 Positive vs Control			
		x2 Value	P value	Odds Ratio	95% CI
238	GG	2.128	0.265	0.574	0.449-0.735
	GA	1.389	0.51	1.714	1.350-2.177
	AA	0.68	1	1.69	1.339-2.132
308	GG	0.216	0.001**	0.762	0.242-2.398
	GA	0.535	0.001**	0.643	0.196-2.108
	AA	4.545	0.069	1.833	1.400-2.401
863	CC	1.637	2.51	2.111	0.667-6.682
	CA	1.797	0.229	0.444	0.134-1.470
	AA	0.181	1	0.643	0.083-4.981
857	CC	0.658	0.506	1.714	0.463-6.351
	CT	1.107	0.454	0.462	0.107-1.988
	TT	0.059	1	1.357	0.115-16.047

Discussion

Adult respiratory distress syndrome is one of the major causes of mortality in pandemic Influenza A H1N1 infection. Analysis of the immune mediators involved in host responses to the virus may elucidate the pathogenic events leading to poor outcomes. T helper-1 (IFN-γ, TNF-α, IL-15) and Th-17 (IL-8, IL-9, IL-17, IL-6) cytokine responses are reported as hallmark of respiratory pathology following infection of H1N1 [4]. These cytokines not only promote antiviral immunity but also contribute to inflammation of respiratory tract by recruitment of neutrophils and mononuclear cells to the site of the infection.

Unlike seasonal influenza A, which replicates mainly in the upper respiratory tract, it has been found that the pandemic H1N1 virus has the ability to replicate in the lower respiratory tract as well. ARDS with postmortem findings of diffuse alveolar damage, reactive hemo-phagocytosis and lymphoid atrophy in the deceased patients were compatible with a cytokine storm as has been reported in H5N1 infection [13,14].

Circulating TNF-α levels and their genetic associations through gene polymorphisms have been correlated in many diseases. The commonly held view is that genetic factors affect TNF-α level. The location of TNF locus is within the class III region of the human major histocompatibility complex (MHC) on chromosome number 6. Recent studies have elucidated the genetic alterations in the TNF- α locus to be involved directly in high TNF-α production. Several polymorphisms have been identified inside the TNF-α promoter positioned at (relative to the transcription start site) -1031(T/C), -863(C/A), -857(C/A), -851(C/T), -419(G/C), -376(G/A), -308(G/A), -238(G/A),-162(G/A), -49(G/A). Among these variants, a polymorphism that directly affects TNF-α expression is located at nucleotide position-308.

A single-base polymorphism within the promoter of the gene of TNF-α results in 2 allelic forms, one in which guanine defines the common allele (TNFA*1) and the other in which guanine is substituted by adenosine forms the rarer allele (TNFA*2) at position-308. The presence of the rarer TNFA*2 allele has been found to correlate with enhanced spontaneous or stimulated TNF-α production both *in-vitro* and *in-vivo* [15].

In parasitic infections, the 308* A allele has been associated with a several fold increase in risk of cerebral malaria with risk for development of serious neurological consequences [16]. The 308*A allele was also reported with the most severe form of mucocutaneous leishmaniasis.

Several studies have investigated the association between TNF alleles and viral infections. The 238*A allele was associated with chronic infection with hepatitis B virus [10]. Similar reports are available for hepatitis C virus infection. Study conducted by Pujhari *et al.* showed that disease severity and disease progression of Japanese encephalitis was associated with the 308 and 863 alleles [11].

As the study on significance of the -238(G/A), -308(G/A), 857(C/A), -863(C/A) alleles of TNF-α gene with the susceptibility to H1N1 infection is neglected entity so far. The single nucleotide mutation at -238 and -308 positions of TNF-α promoter has been found to predispose to higher TNF production [15]. A point mutation from C to T at -857 possessed higher transcription due to the change in the binding stability by variant allele whereas polymorphism at position -863C/A in the promoter region has been reported to be associated with reduced TNF-α promoter activity and lower plasma TNF levels [10, 16]. Based on these observations it was hypothesized that carriage of these SNP alleles may be associated with increased susceptibility to infection with the 2009 H1N1 infection in Indian population. However, in the present study, in patients with influenza A pandemic H1N1 virus infection, TNF-α promoter gene polymorphisms at -308(G/A) was significance in patient group in comparison to healthy controls.

The significance of this finding was underscored by the fact that the number of enrolled patients is small compared with the high frequency of 2009 H1N1 influenza cases. As the SNPs are subjected to change along with the different geographic regions, so the linkage between the - 238 A, -308 A, -857 T and -863 A SNPs and H1N1 infection should be correlated with authentic studies.

Conclusion and Recommendations

From the study we concluded TNF-alpha 308G/A has been shown to be associated with elevated TNF- alpha transcriptional activity. As per the literature search, this is the first study to identify the role of TNF- alpha promoter in Influenza Pdm/09 infection. Our results shows that subjects with -308A are more vulnerable to the severe form of Influenza Pdm/09 infection. This study will help governments to make valuable decisions in allocating scarce resources and providing strategies to limit the spread of influenza as well as severity of the influenza virus.

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Competing Interest

The authors declare that they have no competing interests.

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