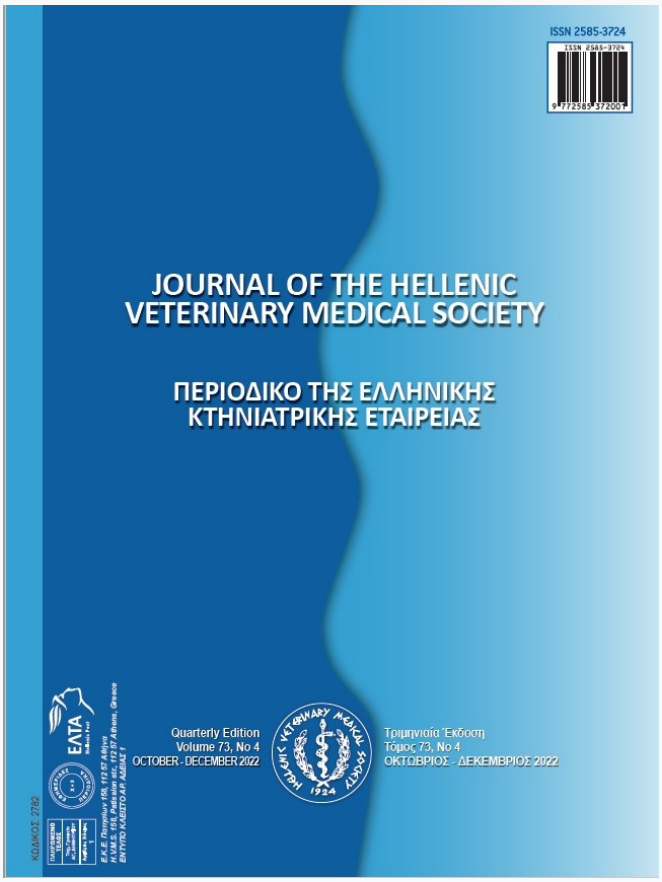
Journal of the Hellenic Veterinary Medical Society

Vol 73, No 4 (2022)



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doi: 10.12681/jhvms.28174

To cite this article:

Malik, K., Zahoor, I., Khalique, A., Sahota, A., & Basheer, A. (2023). Expression profiling of IL-1β, IL-6 and IL-8 genes in lung tissues of Aseel, Crossbred Naked neck, and White Leghorn chicken challenged with H9N2 Influenza virus. *Journal of the Hellenic Veterinary Medical Society*, *73*(4), 4873–4880. https://doi.org/10.12681/jhvms.28174

J HELLENIC VET MED SOC 2022, 73 (4): 4873-4880
ΠΕΚΕ 2022, 73 (4): 4873-4880

Research article
Ερευνητικό άρθρο

Expression profiling ofIL-1β, IL-6 and IL-8 genes in lung tissues of Aseel, Crossbred Naked Neck, and White Leghorn chickenschallengedwith AvianH9N2 Influenza virus

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ABSTRACT: Aseel and Naked neck are major chicken breeds of the tropics and are well-known for their thermotolerance and robustness. However, both of them especially Aseel are very susceptible to Avian Influenza (AI) which causes huge mortalities.The role of cytokines in the pathology and severity of the disease caused by the endemic strain (H9N2) of AIV in thesebreeds remained thus far unclear.The aim of this study was to investigate the effects of H9N2 AIV on the expression level of IL-1β, IL-6, and IL-8 in the lung tissues of Aseel, crossbred Naked Neck, and White Leghorn (WLH). In total 60 birds, 20 from each breed, were used in this study, whereas 30 birds (10 from each breed) were challenged intranasally with the H9N2 virus with a concentration of 10^6 EID_{50} at 6wk of age, and the other half were treated as control. The lung tissues were sampled at the 5th day post-infection to study the differential expression of IL-1β, IL-6, andIL-8 using qRT-PCR. Our data revealed significant differences (P<0.001) in the gene expression levels among all the breeds in response to the viral challenge.It was also observed that after exposure tothe H9N2 virus, Aseel birds showed the highest increase in their expressions of interleukin (IL-1 β, IL-6, and IL-8)genes, followed by Naked Neck, and WLH, respectively suggesting greater susceptibility of Aseel to AIV compared with other breeds. Moreover, these results are in agreement with the severity of disease and incidence of mortality caused by AI in these breeds.

Keywords: Interleukin genes expression, Avian influenza virus (H9N2), Aseel, Naked Neck, White Leghorn

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Date of initial submission: 07-10-2021
Date of acceptance: 04-04-2022

INTRODUCTION

Avian influenza is one of the devastating diseases of poultry that cause huge mortalities and great economic losses to the producers every year. The effect of this disease is not only limited to chicken but covers a wide range of hosts including turkey (Jimenez-Bluhm et al., 2019), geese (Alexander, 2007), quail (Wan & Perez, 2006), pig(Bourret, 2018), equine (Crawford, 2005)and human(Chiaretti et al., 2013); a fact that signifies zoonotic aspect of this disease (Peiris et al., 2001). In spite of spending millions of dollars on vaccination and medication the disease is present in many parts of the world with its full vigor and causing huge economic losses (Çakır et al., 2017). The disease is caused by type A Avian Influenza viruses which belong to the Orthomoxyviridae family. Type A influenza viruses (AIV) are further sub-divided into 18 hemagglutinin (HA: H1-H18) and 11 neuraminidase (NA: N1-N11) subtypes(Tong et al., 2013). The genomic attire of this virus indicates that its genome is a segmented single stranded negative sense RNA(Alexander, 2007).Based on the extent of severity of disease, theAIVis also categorized as low pathogenicity Avian Influenza (LPAI)virus; known to cause disease of mild intensity (Belser et al., 2009), and high pathogenicity Avian Influenza (HPAI); known for its high virulence and devastating outcomes. The flocks challenged withHPAI are at stake of 90-100% mortality due to severe respiratory distress, neurological signs and multi-organ failure (Chmielewski & Swayne, 2011) while birds falling victim to LPAI often goes undetected with mild symptoms such as drop-in egg production and ruffled feathers (Reynolds, 2006).

Once the birds are inflicted with the avian influenza virus, their immune system responds to this calamity by producing certain different types of cytokines (interleukins, interferons, and tumor growth factors) which perform many functions in addition to mediating pro- and anti-inflammatory responses against this virus (Betakova et al., 2017). These cytokines bind to ligand specific receptors and transfer the message to the cell resulting in initiation of a cascade of signal transduction and secondary messenger pathways. IL-1β is known to perform awakening of immune responses during the acute phase (Dinarello, 2018) which further results in stimulation of macrophages and T lymphocytes and production of other cytokines and chemokines mediators (Kaiser, 2004). Likewise, another cytokine, interleukin-6 (IL-6),plays vital role in hematopoiesis in addition to immune regulation and the release of inflammatory serum protein such as amyloid A, C reactive protein (CRP) and α-1 trypsin in mammals (Khalil & Al-Humadi, 2020; Kishimoto, 2010).

Several studies had reported the upregulation of IL-1β, IL-6, IFN-γ and Mx1 genes in the lung tissues of chicken in response to H9N2 challenge(Lee et al., 2013; Reemers et al., 2009). Likewise, the increased expression of IL-6, IL-1β, IFN-β, IFN-γ had also been reported in peripheral blood mononuclear cells (PBMC) of chicken compared with ducksin response to challenge of an LPAI (H11N9)(Adams et al., 2009). However, the challenge with HPAI virus had also been observed to cause the upregulation of pro-inflammatory cytokines including IL-1β, IL-6, IFN-β, IFN-γ, MDA-5 and TLR-3 in the lungs, brain and spleen of chicken (Cornelissen et al., 2013).Some studies had compared the response to HPAI and LPAI viruses and observed increased expression of IFN-α, IFN-β, IL-1β, IL-6, IL8L1, IL8L2, CCL5, CXCL1, CCL20, K203, SCYA4, and TNF-α in DF-1 cell lines (Luo et al., 2018), an upregulation of IL-6, IL-8 and IL-1β in chicken lungs(Rebel et al., 2011), and an upregulation of IL-6 and IL-10 in chicken lung tissues compared with ducks (Kuchipudi et al., 2014)in response to both (LPAI and HPAI) viruses.The current study was designed to determine the differential expression of IL-1β, IL-6, and IL-8 (also known as neutrophils attracting factor) in response to H9N2AI virus (endemic in Pakistan and surrounding countries) in Aseel and crossbred Naked Neckcompared with White Leghorn chickens because the indigenous breeds are considered more immune to the prevalent infections compared with commercial breeds (Dessie et al., 2011).The Aseel birds are known for their vigor, aggressive behavior, greater body size and weight, whereas both Aseel and Naked neck are famous for their thermotolerance, and robustness but are observed to be susceptible to Avian influenza which causes huge mortalities and massive economic losses to the rural farmers each year.

MATERIALS AND METHODS

Ethical statement

The study was conducted after getting ethical approval (173/FVS) from the “Biosafety and Ethical Committee” of University ofVeterinary and Animal Sciences, Lahore, Pakistan

Birds and husbandry

In this study, a total of 150, 50 day-old chicks of each of the Aseel, Naked neck (NN) and White leghorn (WLH) breed were placed in an already prepared brooding room and reared together up to 6wk of age. Before the placement of chicks, the room was disinfected with Virkon S® with a concentration of 1% as per the manufacturer's recommendations. All the experimental birds were managed on a floor covered with litter (4-5 inches thick layer of rice husk, covered with paper during the first week of brooding), and ad-libitum fresh drinking water was provided using automatic bell-shaped drinkers. The chickens were fed acommercial layer starter crumbs diet, formulated according to NRC standards (1994) and feed was offered twice a day in manual feeders. All the possible biosecurity procedures were adopted to prevent any sort of pathogen exposure to the chicks. At the age of 6 weeks, 60 birds (20 birds/breed) were randomly selectedin order to give the viral challenge and were equally divided into treatment (n=10 chicken/breed) and control (n=10 chicken/breed) groups. Hence, each group consisted of a total of 30 birds, with 10 birds of each of the three breeds. After that the treatment group was moved to another facility about 1 Km away from the initial brooding and rearing site in order to give them the challenge of H9N2 Avian Influenza virus. The new facility was also disinfected with Virkon S® using the same protocol and concentrations as described above.

Viral challenge and Sampling

The H9N2 (LPAI) strain of AIV was used in this study, which was obtained from the Microbiology Department of the University of Veterinary & Animal Sciences, Lahore, Pakistan. At the age of 43 days, each bird of the treatment group was intra-nasally given a 0.2 ml of 10^6 EID_{50} of AIV. However, other half of the birds (10 birds per breed)was treated as control. The chickens were humanely killed and sampled 5-days post infection (dpi). After killing and defeathering of chickens, body cavity was opened making sure the blood vessels and surrounding tissues are not damaged. After the successful opening of cavity, air sacs were damaged manually with forceps and 2 lung tissues (0.5gm each) per bird were taken out. After weighing lung tissues were put into labeled cryotubes which were snap frozen in liquid nitrogen. Before initiation of sampling all the biosafety measures were taken into account. All the surgical equipment used for sampling were prior autoclaved and after sampling of each bird the equipment were sprayed with RNase Zap® and wiped out with sterile tissue papers.Even then different set of equipment was used for birds of each breed. After successful sampling, the killed chickens were placed in a ditch with multiple layers of limestone and mud one after the other and were buried under the mud to ensure that no aerosol transmission of pathogen from the killed birds was possible.

Total RNA isolation and cDNA preparation

RNA was extracted from the (~100mg) frozen tissue of thelung after thawing using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) methodfollowed by phenol-chloroform phase separation. The RNA was precipitated using isopropanol and pellet was washed using 80% ethanol. After 15-20 minutes of drying, 100µl of nuclease-free water was added to each tube and vortexed to dissolve the pellet. The extracted RNA samples were quantified by Basic Spectrophotometer® (Eppendorf) and were converted into cDNA for subsequent use inqRT-PCR. For this purpose, 3µl of Reverse Primer (100pmol/µl) was mixed with 8µl of RNA and 4µl of nuclease free water to make a total volume of 15µl. The mixture was incubated at 70°C for 5 minutes and then was rapidly chilled. After first incubation, 2µl of dNTPs, 2µl reverse transcriptase buffer, and1µl of reverse transcriptase enzyme M-MuLV (New England Biolabs®) was added and mixture was incubated at 42° C for 1 hour. Finally, the mixture was incubated at 70^0C for 10 minutes to deactivate the enzyme. The synthesized cDNAs were analyzed on 1% gel electrophoresis and quantified through Basic Spectrophotometer® (Eppendorf).

Quantification of inflammatory interleukins by Real-Time quantitative Polymerase Chain Reaction (RT-qPCR)

SYBR-Green based RT-qPCR was performed on Applied Biosystem®(ABI-7500)by following the manufacturer's recommendation. Each sample was run in duplicate to minimize the chances of error. All target genes with specific primers (Table 1) were normalized against endogenous reference gene (GAPDH) and analyzed in one plate for RT-qPCR. The master mix for RT-qPCR was prepared for each gene with following reaction components viz., 10µl SYBER Green qPCR SuperMix, 0.6µl forward primer, 0.6µl reverse primer,6.8µl nuclease free water and 2µl template cDNA to make total volume of 20µl. Mastermix was filled in 96-well plate which was sealed and centrifuged before placing into qPCR machine. The

Table 1. Primer pairs along with amplicon size and accession number

Gene	Primer Sequence	Product Size (bp)	Accession No.
IL-1β	F: GCTCTACATGTCGTGTGTGATGAG R: TGTCGATGTCCCGCATGA	80	NM204524
IL-6	F: CCTGTTCGCCTTTCAGACCT R: GGGATGACCACTTCATCGGG	171	EU170468
IL-8	F: ATTCAAGATGTGAAGCTGAC R: AGGATCTGCAATTAACATGAGG	196	DQ393272
GAPDH	F: CCTCTCTGGCAAAGTCCAAG R: CATCTGCCCATTTGATGTTG	200	V00407

results were interpreted as differences in fold-changes between control and treatment groups.

Calculations of expression values and statistical analysis

The ΔΔCt values were calculated on the basis of difference in normalized Ct value (ΔCt) from infected samples to the ΔCt from non-infected samples. The ΔΔCt values were transformed into $2^{-\Delta\Delta Ct}$ value method using glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) as the endogenous reference gene.Logarithmic transformation of data on fold-change values was done before performing the statistical analysis.

In case of statistical analysis,2 x 3 (treatment x breed) factorial ANOVA was employed under the general linear model (GLM) procedure ofGenStat®, version 19, (https://www.vsni.co.uk)to determine significant difference between the Ct values of the lung tissues for the control and target genes. Mean Ct values along with their standard deviations were used to calculate the fold-changes in the expression of all of the three target genes and the following statistical model was used to analyze the data

$$Y_{ijk} = \mu + B_i + V_j + (B_i * V_j) + e_{ijk}$$

Where Y_{ijk} is the dependent variable, μ is the population mean, B_i is the fixed effect of i^{th} breed, V_j is the fixed effect of jth viral treatment, $B_i * V_j$ is the interaction between the i^{th} breed and j^{th} viral treatment, and e_{ijk} is the error.

The model included the breed, viral treatment and their interaction as the factors. However, when the effect of any factor was found statistically significant the means were further compared by using the least significant difference (LSD) test in the GenStat® (version 19).

RESULTS AND DISCUSSION

Clinical signs

Following infection with H9N2 virus, birds of all three breeds started to show clinical symptoms like reduced feed intake, depression and mild ocular discharges at 2-day post-infection (dpi). At 5dpi most of the birds were having clear signs of swollen cyanotic wattles, ocular and nasal discharges and difficult breathing with snoring sounds. However, none of the treated birds died by the end of 5^{th} dpi.

All the treated birds of three breeds showed prominent symptoms of the disease including ocular and nasal discharges with ruffled feathers, swollen head and cyanotic wattles and combs but they were more pronounced in Aseel compared with the other two breeds.These findings were further confirmed by the post-slaughter lesions on various organs such as spleen and liver, which were found inflamed and enlarged in their size in Aseel compared with WLH, and NN.The lungs showed mild enlargement with hyperemia suggesting viral replication in Aseel, whereas the severity of lung inflammation was lesser in other two breeds. These results are in agreement with the findings of Swayne et al. (2007) who observed more congestion and enlargement of lungs along with splenomegaly in breeds more susceptible to AI.

Differential expression of inflammatory cytokines in H9N2 challenged chickens

IL-1β gene

The results of analysis of variance of IL-1β gene showed significant effect of'Breed' ($P<0.001$) and 'Treatment' ($P<0.027$) separately, whereas the Breed x Treatment interaction had non-significant effects ($P=0.348$) (Table 2). The least square means (LSMs) for the interaction of breed and treatment showed that there was significant difference ($P<0.05$) in LSMs of the challenged and control birds of WLH (Table 3). In the case of IL-1β gene, our results showed 14.2, 7.8, and 6.3-folds increase in the expression values

Table 2. Results of analysis of variance for effect of Breed x Treatment on normalized Ct-values of IL-1β, IL-8, and IL-6 gene in Aseel, Naked neck, and WLH chicken

Gene	Source of variation	Degree of Freedom	Sum of Squares	Mean Sum of Squares	P-value
IL-1β	Breed	2	306.438	153.219	<0.001
	Treatment	1	10.573	10.573	0.027
	Breed x Treatment	2	4.372	2.186	0.348
IL-8	Breed	2	121.447	60.724	<0.001
	Treatment	1	0.103	0.103	0.804
	Breed x Treatment	2	56.257	28.128	<0.001
IL-6	Breed	2	152.9449	76.4725	<0.001
	Treatment	1	106.8244	106.8244	<0.001
	Breed x Treatment	2	30.7928	15.3964	<0.001

Table 3. Breed-wise and treatment-wise least square means of normalized Ct-values for challenged and control groups of Aseel, cross-bred Naked neck, and White leghorn chickens

	Aseel			Naked Neck			White Leghorn		
Gene	IL-6	IL-8	1L-1b	IL-6	IL-8	1L-1b	IL-6	IL-8	1L-1b
Challenged	24.8[a]	31.3	24.6	27.2[a]	27.3[a]	27.8	24.1	30.9[a]	29.6[a]
Control	29.4[b]	32.0	25.2	29.7[b]	29.1[b]	28.2	25.1	28.1[b]	31.2[b]

Means with different superscript within the same column represent a significant (P<0.05) difference.

of treated groups of Aseel, NN, and WLH respectively (Figure 1). The increase in the treated groups was significantly greater in all the breeds compared with their respective control; additionally, this increase in expression values was significantly (P<0.05) greater in treated Aseel compared with other two breeds (Figure 1). The greaterfold-change values of the expression of IL-1βin Aseel and NN than WLH suggest that they are more susceptible to AIV than WLH. These findings are in agreement with higher mortality percentages of Aseel and NNin the field caused by AI outbreaks (personal observation). The increased expression of cytokines in native chicken breeds in the current study is suggestive of the fact that though these breeds are resistant to many pathogens but are susceptible to AI virus. Consistent with our results Noah et al. (2003) observed 100-fold more expression of IL-1β in peripheral blood mononuclear cells of chicken compared with duck, and reported that the reduced production of IL-1β in ducks was due to greater expression of an immunomodulator (NS1A). And the pathophysiology of such reduction in expression values was found to be the result of interaction between polyadenylation specificity factor, cellular protein cleavage and NS1A leading to suppression of IL-1β in duck. It is likely that the reduced expression of this gene in WLH might be due to the physiological pathway of NS1A in WLH. Hence, it is speculated that the NS1A pathway might be involved in the reduced expression of IL-1β in WLH compared with Aseel and Naked neck chicken. In another similar study,Adams et al. (2009)evaluated the expression of IL-6 and IL-1β in chicken and duck PBMCs in response to LPAI (H9N11) and observed down-regulation of these two pro-inflammatory mediators of avian influenza. The authors linked this increased expression of genes in chicken to a stronger Th2 response while down-regulation of these genes in duck was linked to a weak Th1 response.

The difference in the fold-change values of genes expression in different hosts challenged with the same virus may be related to the difference in the susceptibility of the hosts. For instance, H9N2 virus used in the present study caused less than 20-folds genes expression of IL-1β (Figure 1) in all three chicken breeds at dpi 5 in lungs, whereas H9N2 strain SD818 used by (Wang et al., 2016) caused almost 30-folds expression in the lung tissues. Surprisingly, Rebel et al. (2011) showed insignificant difference in the induction of cytokines expression either with highly pathogenic or low pathogenic influenza virus within

first 24 hours. Moreover, in agreement with the findings of the current study, levels of IL-6 and IL-1β were remarkably upregulated in the lungs of chicken when infected by HPAI viral strain H5N6 in the study conducted by (Gao et al., 2017).

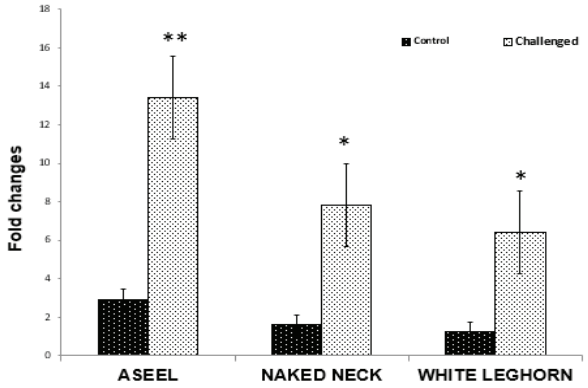

Figure 1. Relative fold-changes in IL-1β expression in Aseel, Crossbred Naked Neck and White Leghorn Layer

IL-6gene

The results of analysis of variance showed that the 'Breed', 'Treatment' and their interactions all had highly significant (P<0.001) effects on the Ct-values of IL-6 in the lung tissues of chickens.The LSD analysis revealed that there was significant difference between the least square means of the challenged and control group of Aseel, and NNchickens (Table 3).However, the fold-change analysis of expression values of IL-6 showed that there was significant (P<0.01) increase in the expression values of treated groups of all the breeds compared with their controls (Figure 2); however, in case of among breed comparison there was no significant difference in the fold-change expression values of IL-6 (Figure 2). IL-6 is one of the major inflammatory mediators and the expression of these cytokines is directly associated with immensity of viral replication, fever, respiratory tract inflammation, and systemic symptoms of the influenza virus (Nguyen et al., 2019; Wang et al., 2016). And its greater expression in Aseel is indicative of its greater susceptibility towards the AIV. Likewise, other respiratory viruses such as SARS virus had also been notoriously linked with the production of IL-6 in the lung tissues (Cheung et al., 2005) which suggests the similar pathological effects of respiratory viruses in the lung tissues of chickens. In agreement with our results, some studies on mice(Wu et al., 2020)and pigs (Czyżewska-Dors et al., 2017) indicated similar results by showing an increased expression of IL-6 and IL-1β in the lung tissues in response to the AI virus. Studies on humans naturally infected with highly pathogenic avian influenza strain (H5N1) had shown the production of pro-inflammatory cytokines in their lungs in comparison to healthy individuals (Thitithanyanont et al., 2010). These studies on humans, and chicken indicated that upon infection of influenza virus both mammalian and avian species employ almost similar kind of response to get rid of the invading pathogen.

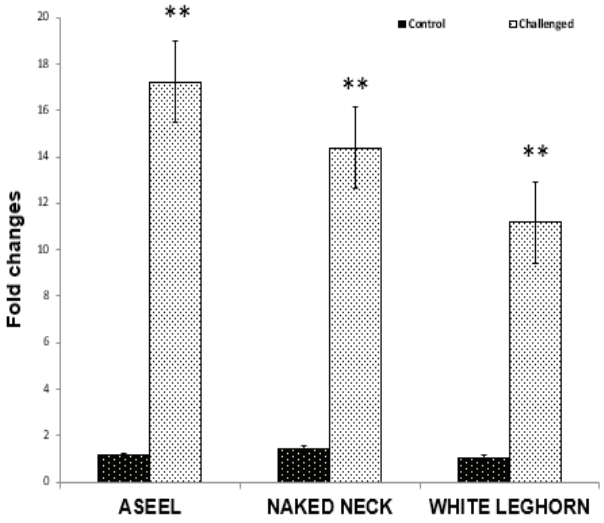

Figure 2. Relative fold-changes in expression of IL-6 in Aseel, Crossbred Naked Neck and White Leghorn chicken

IL-8 gene

The results of statistical analysis showed that 'Breed' and 'Breed x Treatment' interaction had highly significant (P<0.001) effects on the Ct values of IL-8, whereas 'Treatment'showed non-significant effects (P=0.804) (Table 2). Moreover, the post-hoc analysis revealed that there was significant (P<0.05) difference in the LSMs of challenged and control groups of NN and WLH (Table 3). The fold-change analysis of expression values showed that there was significant increase in the expression of IL-8 in the treatment group of all the breeds compared with their respective control groups (Figure 3). Moreover, it was also observed that in case of among breeds comparison, there was significant (P<0.05) post-treatment increasein the expression of IL-8 in Aseel chicken compared with NN and WLH (Figure 3). Our results are similar tothe findings ofBergervoet et al. (2019) who observed increased expressions of IL-8 in macrophages of H9N2 infected chicken. Likewise,Cornelissen et al. (2012)also reported the significantly increased expression of IL-8 in chicken lungs in response to HPAI challenge which

is consistent with the findings of the present study in which IL-8 was seen to be expressed 7-folds in Aseel, whereas this increase was only 5.5- and 4.6-folds for NN and WLH respectively. In agreement with our results,Ku et al. (2014)observed only less (1.5-fold) increase in expression of IL-8 in lung tissues of WLH chicken infected with H9N2 virus. Their results were suggestive of the response of chicken towards LPAI virus in terms of IL-8 expression.

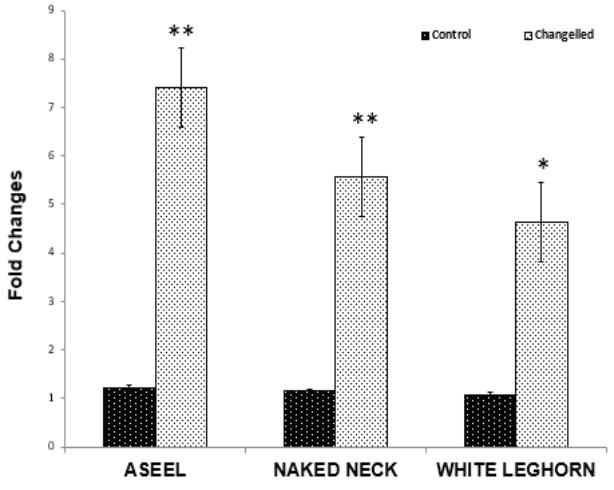

Figure 3. Relative fold-changes in IL-8 expression values in Aseel, Crossbred Naked Neck and White Leghorn chicken

However in contrast withour results,Jiao et al. (2018) could not find any significant difference in the expression of the IL-8 gene in the chickens infected with the H7N9 AI virus. The reason for this no difference in the expression values might be that they did use adult chickens in their study and secondly the H7N9 virus infection is very mild and had not been reported to cause any mortality except for the loss of only 6.5% weight in chicken (Ku et al., 2014).

CONCLUSIONS

This study has demonstrated a general trend of expressions for all of the three genes in lung tissues which were the highest in Aseel followed by crossbred Naked neck and WLH respectively (Aseel >crossbred Naked Neck > WLH) and this pattern of gene expression also coincide with severity of symptoms and postmortem lesions in these tree breeds of chicken.

Correlating with previous findings on the interaction of Influenza virus and cytokines, this study has demonstrated that lungs are the primary site for virus replication and most of the pathology caused by AIV. Upon interaction with virus different breeds responded differently with significantly different fold-changes in the expression of pro-inflammatory cytokines. The maximum expression of these genes in Aseel is indicative of the fact that Aseel possesses greater susceptibility towards Avian Influenza virus. These results are also supported with a greater mortality percentage of Aseel in case of Influenza virus outbreaks in the country compared with the other two breeds. To our knowledge, this is the first study in which expression profiling of some genes is studied in response to any strain of AIV.

ACKNOWLEDGEMENT

The work was generously supported by the Pakistan Agriculture Research Board through a grant no PARB 576. We would like to acknowledge the role of AsadAbbass, Numan Sharif and poultry farm staff for their help in sampling. However, all the technical support was provided by the Animal Genetics and Genomics Lab, Central Laboratory Complex, University of Veterinary and Animal Sciences, Lahore.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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