Research Article

Immune-Related Gene Expression in Response to Different Strains of Egyptian Low Pathogenic H9N2 Infection in Chicken Peripheral Blood Mononuclear Cells

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Abstract | Avian influenza H9N2 represents one of the most important challenges to poultry production worldwide. That is in addition to its ability to induce mortalities and reduce the productivity, it accused to be negatively affecting the avian immune response facilitating the secondary infection. Many reports take into account the genotypic and pathogenic characterization of H9N2 in avian species. However little is known about the impact of different strains on the innate immune response. In the present study, using quantitative real-time PCR, cytokines gene expression were examined in response to infection with two strains of Egyptian H9N2 (namely V3 and RSF/1) in chicken peripheral blood mononuclear cells (PBMC). Hemagglutinin gene sequence analysis of the two strains reveal high similarity with difference close to cleavage site (HARSSR/ GLF) and (PARSSR/GLF) for V3 and RSF/1 respectively. Results of growth kinetics in PBMCs showed that V3 replicated more efficiently comparing to RSF/1 at all-time points. Further, strain V3 induced significant up-regulation of IL-1b and IL-6 at 24 hours post infections (hpi) comparing to RSF/1. However, both strains induced significant up-regulations of interleukin (IL)-8 at 1 and 3hpi and interferon (IFN)-α late 24 and 48hpi. Altogether, our results suggest that the concurrent up-regulation of IFNα, IL-1b and IL-6 with higher viral load could represent an important feature affecting the viral pathogenicity and associated with impairment of innate immune response that in turn facilitate and magnify the effect of co-infections.

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Introduction

Avian influenza subtype H9N2 is a member of the Family Orthomyxoviridae, since its first isolation in the USA in 1966 it has been detected in many countries (Homme and Easterday, 1970). At the last two decades H9N2 cause many out breaks throughout the world, it transe from Asia to the Middle East (Iran, Pakistan, Israel, Jordan, Lebanon, United Arab Emirates and Saudi Arabia) (Nili and Asasi, 2002; Perk et al., 2006) and Africa (South Africa and Tunisia) (Abolnik et al., 2010; Tombari et al., 2011). H9N2 was not detected in Egyptian farms until early 2011(El-Zoghby et al., 2012), since then the virus

detected all over Egypt causing great economic losses especially with co-infection with other viruses or bacteria (Hassan et al., 2016). Not surprisingly that the nucleotide sequencing of the hemagglutinin gene of Egyptian clustered tightly with Israeli and Lebanese strains (Arafa et al., 2012; Kandeil et al., 2014). Phylogenetically the Egyptian strains belong to the A/quail/Hong Kong/G1/1997-like lineage with particular mutations identified in all viral proteins (Arafa et al., 2012).

H9N2 AIV is prevalent in chickens, ducks and other poultry species in addition to Pig (Xu et al., 2004). Human infections with H9N2 viruses were reported in Hong Kong in 1999 as the first avian to- human transmission, a subsequent case was reported in 2003,2008 and 2011 (Butt et al., 2005), also it is reported in Egypt early 2015 in 3 years old boy (WHO, 2015).

In a national surveillance conducted by RLQP (Reference Laboratory for Veterinary Quality Control in Poultry Production, Egypt) during 2012, H9N2 virus was detected in 35 farms out of 200 farms that have respiratory problems. 91% of these cases were coinfected with other bacterial and viral respiratory pathogens. While, only 9% out of these positive cases has only H9N2 virus (data not published). And recently the mixed infection with H9N2 in broiler farms become more predominant that induced devastating effects on the local poultry industry (Hassan et al., 2016).

Many records highlighted the ability of predisposing infection with H9N2 to enhance the effect of the secondary bacterial infection as *Staphylococcus* sp. *E.coli*, *Ornithobacterium rhinotracheale* and *Mycoplasma* (Azizpour et al., 2014; Bano et al., 2003; Pan et al., 2012; Popy et al., 2012; Roussan et al., 2015; Sid et al., 2015) and enhanced the morbidity and mortality when accompanied with infectious bronchitis (IB) infection (Hassan et al., 2016). The mechanism of this enhancement still not well understood. However, different hypotheses could explain this phenomenon. First, through mechanical damage of the ciliated cells and the epithelial lining the respiratory tract which in turn diminish the microbial clearance and enhance pathogens attachments and colonization (Bakaletz, 1995; Wilson et al., 1996), second, functionally, via alteration of the innate immune response including impairment of phagocytic activity (Debets-Ossenkopp et al., 1982; Engelich et al., 2002; Navarini et al., 2006), and/or overproduction of inflammatory cytokines, which enhance the pathogenicity of the invading pathogens (Beadling and Slifka, 2004; Speshock et al., 2007; Van Der Sluijs et al., 2006). Altogether, despite the low pathogenicity nature of H9N2 but it can cause a serious problem especially if complicated with other bacterial and viral diseases. This highlights the importance of figure out the effect of H9N2 on the innate immune response. H9N2 were detected and isolated from PBMCs which suggest that the low pathogenic avian influenza virus (LPAI) subtype H9N2 virus might be disseminated throughout the body of the chicken through the blood or lymph vessels (Post et al., 2013). In the present study PBMCs from the chicken were used for studying the avian innate immune response to infection with two genetically distinct H9N2 strains by comparing cytokines gene expression and viral replication in light of genetic and phylogenetic analysis.

Material and Methods

Viruses

Two avian influenza A subtype H9N2 viruses were provided by the Reference Laboratory for Veterinary Quality Control on Poultry Production Giza, Egypt (RLQP). The first (A/chicken/Egypt/123140V /2012 (H9N2)) with accession number KJ781210 and so named (V3), the second (A/chicken/ Egypt/1433RSF/2014(H9N2)) with accession number KU296203 and so named (RSF/1). Two viruses propagated in specific-pathogen-free (SPF) embryonated chicken eggs and EID50 were calculated according to stander procedure (OIE, 2014; Reed and Muench, 1938). The collected allantoic fluid was tested for the presence of AIV-H5, AIV-H7, NDv and IBv by polymerase chain reaction. Briefly, RNA was extracted from allantoic fluid using QIAamp viral RNA extraction kit (QIAGEN) and amplified using Quantitect probe RT-PCR kit (QIAGEN). Primers, probes sequences and thermal profiles previously described (Callison et al., 2001; Slomka et al., 2007; Wise et al., 2004).

Gene Sequencing

Viral RNA was extracted from allantoic fluid using QIAamp viral RNA extraction kit and amplified using Qiagen OneStep RT-PCR (QIAGEN) according to manufacturer instruction, and primers sequencing to amplify full haemagglutinin nucleotide (HA) gene and thermal profile (available upon request). PCR product subjected to electrophoresis and the specific band excised and then purified by a QIAquick Gel Ex-

traction Kit (Qiagen). Full HA nucleotide sequencing was carried out using Big Dye Terminator kits (version 3.1; Applied Biosystems, Foster City, CA) and figured out on 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). The retrieved sequences assembled by the Lasergene DNASTAR (DNASTAR Inc., Madison, WI). The assembled sequences of the two strains used in the present study in addition to Egyptian H9N2, ancestral and regional strains with full HA gene nucleotide sequences were retrieved from gene bank database, MEGA 5 software used for alignment and construction of phylogenetic tree using Kimura's two-parameter distance model and 1000 bootstrap replicates (Tamura et al., 2011). Tree was viewed and edited using FigTree v1.4.2 software (http://tree.bio.ed.ac.uk/software/figtree/).

Chicken Peripheral Blood Mononuclear Cell Isolation, Culturing and Infection

PBMCs were purified from heparinized blood using Ficoll-Paque (GE healthcare, Sweden) according to the manufacturer instruction. PBMCs suspended in complete RPMI media (Gibco, Carlsbad, CA, USA) with cell density approximately 10^7 cells/ml and cultured in 6 wells tissue culture plates and then overnight incubated at 37°C in 5% CO_2 incubator. Cells were infected with V3 and RSF/1 strains separately in four infection doses 1, 0.5, 0.1, 0.01 EID_{50} /cell.

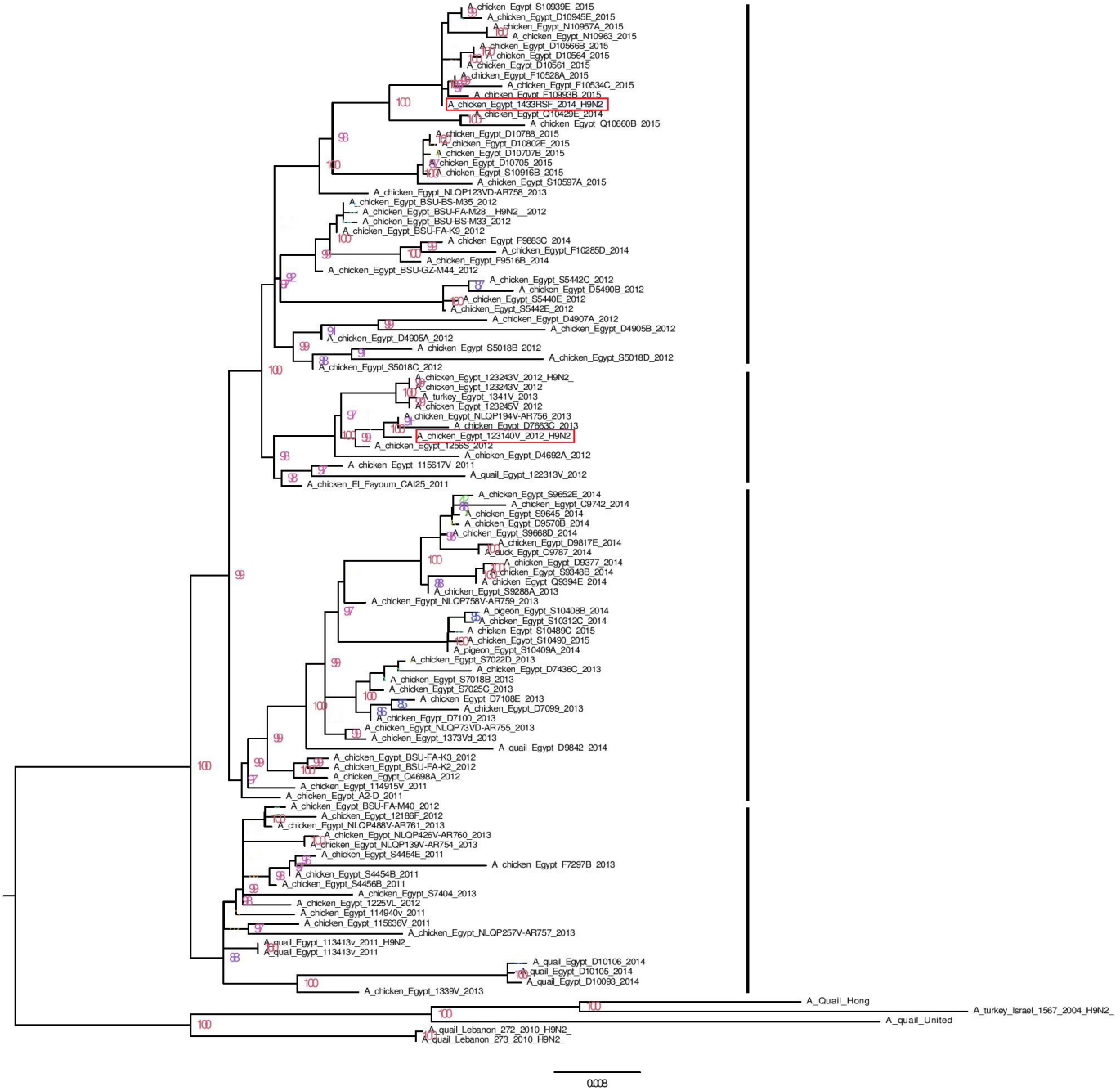

Figure 1: *phylogenetic relationship of the HA nucleotide sequences of V3 and RSF/1 to Egyptian H9N2 strains and G1-like ancestor strains (based on the full HA nucleotide sequences)*

Then the infection dose 0.1 EID_{50}/cell was chosen to investigate the cytokines gene expression of PBMCs in response to infection with H9N2 V3 and RSF/1 strains, cells and supernatant collected at 1, 3, 6, 24 and 48 hpi.

Extraction of RNA and Quantitative Real Time PCR

Total RNA was extracted from the cells and its supernatant of infected and control cells at each time point using the RNeasy mini RNA Purification kit (QIAGEN) according to the manufacturer instructions. All RNA samples were checked using NanoDrop® Spectrophotometer ND-1000 (Thermo Scientific, USA). Cytokines mRNA gene expression of the cells after infection quantified relatively to the negative control after normalized against 28 sRNA, while virus growth curve was determined by absolute quantification using stander curve for a known titer strain. In both cases, quantitative real time RT-PCR was performed using Quantitect probe RT-PCR (QIAGEN) according to the manufacturer instruction, thermal profile, primers and probes sequences for IL-1b, IL-6, 28-S and IFN-γ previously described (Kaiser et al., 2000), IL-8 (Kogut et al., 2003) IFN-α (Eldaghayes et al., 2006), and AI-H9 (Shabat et al., 2010). qRT-PCR was performed using a 7500 Real-Time PCR System (Applied Biosystems).

Statistical Analysis

Cytokines gene expression represented by fold changes which calculated by −ΔΔCT method (Livak and Schmittgen, 2001). Samples collected at each time point in triplicate to calculate the stander error for treated and non-treated cells at each time point. The student T-test was used to determine significant differences between fold change values of the treated cells with V3 and RSF/1 viruses at each time points and the control group.

Results

Hemagglutinin Phylogenetic and Sequence Analysis

Based on phylogenetic analysis all Egyptian strains were belong to G1-like Eurasian sub-lineages (A/quail/Hong Kong/G1/97-like) (Fusaro et al., 2011) and clustered tightly with Israeli and Lebanese strains in group B. The Egyptian strains could be further divided into 4 distinct groups (bootstrap value ≥ 99) could be named as B1 – B4 (Figure 1). Indeed, this differentiation does not depend on time collection, region, host, season or even rearing practicing.

The sequence analysis of the full HA gene of the two selected viruses showed that nucleotide and deduced amino acid sequences similarities were 97 and 98%, respectively. V3 and RSF/1 strains have nucleotide homology about 90 and 89% and amino acid homology 96 and 91%, respectively, with the ancestor strain (A/Quail/Hong Kong/G1/97 (H9N2)). The detailed amino acid comparison of the HA genes of both strains with the ancestor H9N2 viruses in addition to other regional strains were summarized in (Table 1).

Growth Curve

The used strains were checked for the presence of AI-H5,AI-H7, NDv and IBv before the in-vitro study using real-time RT-PCR and the results revealed the absence of these pathogens. Viral titer at the culture supernatant at each time point were checked and the results revealed that strain V3 more efficiently replicated comparing to RSF/1 by approximately 2 folds at all-time point. For both strains, there is no significant increase in the virus titer with the time (Figure 2).

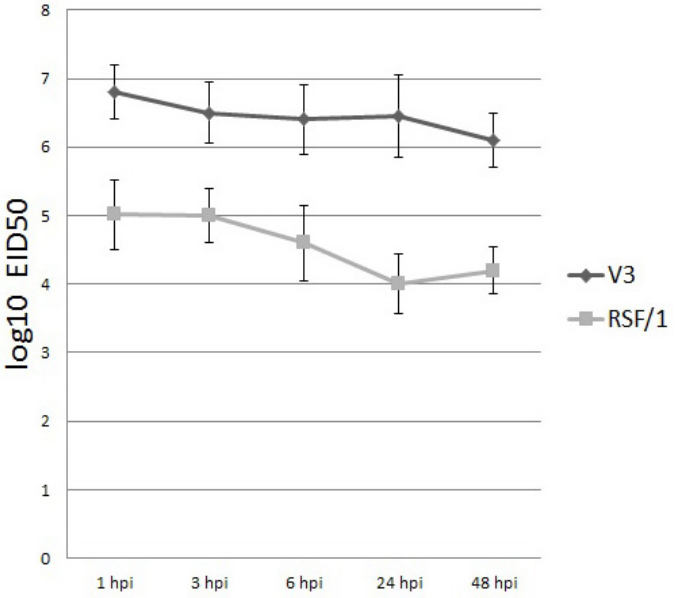

Figure 2: *Viral titer of V3 (black column) and RSF/1 (gray column) in PBMCs culture supernatant at different time points. Viral titer measured by quantitative RT-real time PCR and expressed by log_{10} EID_{50}. Data showed are the mean with error bar calculated from three replicates at each time point*

Differential Cytokines Gene Expression

Early after infection (1 and 3 hpi), the expression of IL-8 significantly upregulated with both strains infection. However, it tends to be a higher expression with V3 comparing to RSF/1 infection. Concerning to IL-1b, IL-6, IFNα and IFNγ it showed no significant difference comparing to non-infected negative control cells (Figure 3).

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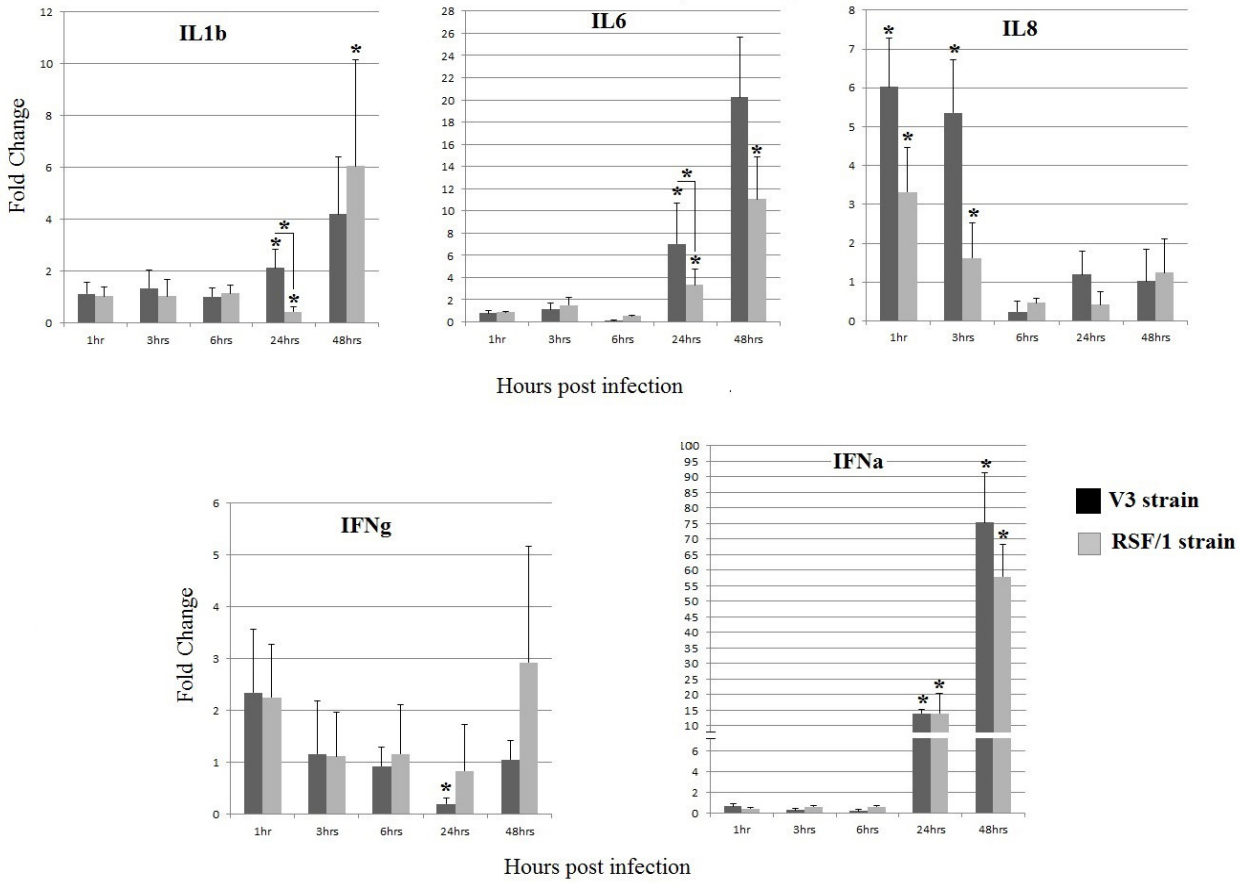

Figure 3: *Cytokines gene relative expression in PBMCs cell culture infected with V3 strain (black column) and RSF/1 strain (gray column): *P < 0.05, asterisk over error bar represent significant difference between infected and non-infected cells. While asterisk over horizontal line represent significant difference between both strains. Data showed are the mean with error bar calculated from three replicates at each time point*

Late after infection (24 and 48hpi), IFNα significantly upregulated without significant difference between both strains. In contrast, the expression of pro-inflammatory cytokines IL-1β and IL-6 were significantly higher in the case of V3 comparing to RSF/1 infection at 24hpi (Figure 3). While at 48hpi both strains induced upregulation of IL-1b and IL-6 that was significant only in case of RSF/1 infection. In all time points, IFNγ showed no significant difference comparing to the non-infected cells except at 24 hpi it is significantly downregulated upon infection with V3 strain. In general, V3 showed higher capacity in the induction of cytokines and chemokine expression as compared with RSF/1 with the exception of IFNγ expression pattern.

Discussion

Most of the LPAI-H9N2 infections cause no or little clinical signs with a drop in egg production unless it is complicated with other pathogens (Haghighat-Jahromi et al., 2008; Naeem et al., 1999). Egypt in the last few years experienced many outbreaks of H9N2 with high mortality rate and dramatic diminish of the productivity (Hassan et al., 2016). And with taking in mind the recorded low evolution rate, absence of reassortment evidence and the phylogenetic homogeneity of Egyptian H9N2 viruses (Kandeil et al., 2014; Naguib et al., 2015), the lack of vaccination against H9N2 with its potential immunosuppressive effect accused to be the main reason for the high prevalence of mixed respiratory infection (Hassan et al., 2016). Mononuclear cells represent the main source of cytokines and chemokines production in response to influenza A virus infection (Kaufmann et al., 2001). So the present study was designed to investigate the effect of two genetically distinct H9N2 strains infection on cytokines gene expression profiles of PBMCs that could serve as a start point study the host-virus interaction.

The two strains used in the present study have typical glycosylation sites, antigenic sites, receptor binding pocket, receptor binding site (Table 1) and possessed H191 and L234 (H9 numbering), viruses with these substitutions showed preference to 2-6α sialic acid receptor (human-like) (Matrosovich et al., 2001) and higher replication in human cells (Matrosovich et al., 2004).

Table 1: *Amino acid comparison of the HA gene of V3 and RSF/1 strains used in the present study with the ancestor G1-like strain and other regional strains*

	Glycosylation site								Antigenic site			Receptor pocket			Cleavage site	receptor binding site
									Site I	Site II	Over lapping site	left edge	binding site	right edge		
Amino acid (H9N2 numbering)	29	105	141	206	218	298	305	492	143, 166, 170	155, 201, 234	187, 206	232-237	106, 109, 161, 163, 191, 198, 202, 203	146-150	333-341	166,191,197,198,232,234,235,236,399
A/quail/ Hong Kong/ G1/97	NST	NGT	NVT	NDT	NRT	NST	NIS	NGT	TSP	FNL	TN	NDLQGR	G,Y,W,T,H,E,L,Y	GISRA	PARSSR/GLF	S,H,T,E,N, L,Q,G,K
A/turkey/ Israel/1567/ 2004 (H9N2)	NST	NGT	NVT	**T**DT	NRT	NST	NIS	NGT	TSP	FNL	T**T**	N**G**LQGR	G,Y,W,T,H,**A**,L,Y	G**T**S**K**A	PARSSR/GLF	S,H,T,**A**,N, L,Q,G,K
A/quail/ Lebanon/ 272/ 2010 (H9N2)	NST	NGT	NVT	**T**DT	**D**RT	NST	NIS	NGT	TNP	FNL	T**T**	N**G**L**I**GR	G,Y,W,T,H,**V**,L,Y	G**T**S**K**S	PARSSR/GLF	**N**,H,T,**V**,N, L,**I**,G,K
A/quail/ UAE/ D1556/ 2011 (H9N2)	NST	NGT	NVT	NDT	**D**RT	NST	N**V**S	NGT	T**R**P	FQQ	**S**N	N**G**Q**F**GR	G,Y,W,T,H,**I**,L,Y	G**T**S**S**S	PARS**R**R/GLF	**R**,H,T,**I**,**G**,**Q**, **F**,G,K
A/chicken/ Egypt/ 123140V/ 2012 (H9N2) (V3 strain)	NST	NGT	NVT	**T**DT	**D**RT	NST	NIS	NGT	TNP	FNL	T**T**	N**G**L**I**GR	G,Y,W,T,H,**A**,L,Y	G**T**S**K**S	**H**ARSSR/ GLF	**N**,H,T,**A**,N,L, **I**,G,K
A/chicken/ Egypt/ 1433RSF/ 2014 (H9N2) (RSF/1 strain)	NST	NGT	NVT	**T**DT	**D**RT	NST	NIS	NGT	TNP	FNL	T**T**	N**G**L**I**GR	G,Y,W,T,H,**A**,L,Y	G**T**S**K**S	PARSSR/GLF	**N**,H,T,**A**,N,L, **I**,G,K

Concerning the cleavage site motif sequences indicating low pathogenicity for V3 and RSF/1 strains with different pattern HARSSR/GLF and PARSSR/GLF, respectively. Parvin et al. (2015) concluded that (A/chicken/Dubai/ F5/2013) with HARSSR/GLF cleavage site showed higher replication profile comparing to (A/chicken/Saudi Arabia/R61/2002) with PARSSR/GLF cleavage site in embryonated chicken egg, although the later contain Q at position number 234 (H9 numbering) which is a typical avian H9N2 signature (Wan and Perez, 2007). This goes in context with our finding, as V3 strain grow more efficiently in PBMCs comparing to RSF/1 in all time points.

At the Early stage after infection (1 and 3hpi) IL-8 gene expression upregulated significantly upon infection with both strains. IL-8 is a proinflammatory cytokine produced at the early stage of infection lead to influx of heterophils to the site of infection (Kogut, 2002) that mediated acute inflammation and tissue damage (Kogut et al., 2003). Later both strains induced significant upregulation of IL-1β and IL-6. IL-1β is a proinflammatory cytokine that obviously increased at the site of inflammation with increaseing tissue damage and then increase the release of the other inflammatory mediators, such as IL-6, IL-8 and TNF-a (Liu et al., 2016) that subsequently promote the inflammation response implicated in pulmonary tissue pathology (Kim et al., 2015) and therefore enhance tissue damage associated with H9N2 infection severity (Wang et al., 2016). Furthermore, IL-6 is one of the main inflammatory mediators that its expression positively correlated with viral replication, fever and enhanced systemic form of influenza (Kaiser et al., 2001). Taken together, it was not a surprising significant higher expression of IL-1b and IL-6 upon infection with V3 strain comparing to RSF/1 at 24hpi if we take in mind that the highest difference in the viral load between both strains reported at 24hpi (Figure 3). In a previous record, higher IL-1b, IL-6 expression in lung, spleen and cecal tonsil were correlated with higher viral titer (Guan et al., 2015).

Among the key findings of the current study was that the INFα expression. It is widely accepted that influenza virus antagonize type I IFN production via various mechanism (Rajsbaum et al., 2012). However, no correlation between the viral pathogenesis and the level of type I IFN could be confirmed (Penski et al., 2011). Our finding showed significant upregulation of IFNα upon infection with both strains and tend to be higher expressed with V3 comparing to RSF/1infection at 48 hpi. Such upregulation upon infection with H9N2 previously recorded (Wang et al., 2016). IFNα is known to be a key antiviral cytokine and pretreatment with IFNα significantly reduce influenza replication (Jiang et al., 2011). However, excessive IFNα signaling due to influenza infection and/or treatment with IFNα of influenza infected mice associated with more lung damage, proinflammatory cytokines, and lung infiltrating inflammatory cells (Davidson et al., 2014). This support our finding of the concurrent higher expression of IFNα, IL1β, IL-6 and the higher viral load could be the cause of innate immune response dysregulation upon infection with H9N2 infection and could be the cause of the facilitating the secondary infection, these findings need to be confirmed *in vivo*.

Conclusion

In conclusion, the present study supports the claim that the higher viral replications with dysregulation of cytokines. Further, the persistence of LPAI-H9N2 infection and shedding could be attributed to the persistence of tissue damage due to the failure of the inflammatory response to control the viral replication.

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Conflict of Interest

Authors declare that they have no conflict of interest.

Authors' Contribution

A. Samy conceived the study; A. Samy and W. Mady performed the research; A. Samy, W. Mady, N. M. Haggag and S. H. Hamoda were involved in drafting the work and revising it critically for important intellectual content. A. Samy wrote the manuscript. And A. Samy, W. Mady, N. M. Haggag, S. H. Hamoda, I. N. El Shamy and M.K.Hassan compiled the final approved version to be published

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