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Genome-wide gene expression pattern underlying differential host response to high or low pathogenic H5N1 avian influenza virus in ducks

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Summary. – The differences in the influenza viral pathogenesis observed between different pathogenic strains are associated with distinct properties of virus strains and the host immune responses. In order to determine the differences in the duck immune response against two different pathogenic strains, we studied genome-wide host immune gene response of ducks infected with A/duck/India/02CA10/2011 and A/duck/Tripura/103597/2008 H5N1 viruses using custom-designed microarray. A/duck/India/02CA10/2011 is highly pathogenic virus (HP) to ducks, whereas A/duck/Tripura/103597/2008 is a low pathogenic (LP) virus strain. Comparative lung tissue transcriptome analysis of differentially expressed genes revealed that 686 genes were commonly expressed, 880 and 1556 genes are expressed uniquely to infection with HP and LP virus, respectively. The up-regulation of chemokines (*CCL4* and *CXCR4*) and IFN-stimulated genes (*IFITM2, STAT3, TGFB1* and *TGFB3*) was observed in the lung tissues of ducks infected with HP virus. The up-regulation of other immune genes (*IL17, OAS, SOCS3, MHC I* and *MHC II*) was observed in both infection conditions. The expression of important antiviral immune genes *MX, IFIT5, IFITM5, ISG12*, β-defensins, *RSAD2, EIF2AK2, TRIM23* and *SLC16A3* was observed in LP virus infection, but not in HP virus infection. Several immune-related gene ontology terms and pathways activated by both the viruses were qualitatively similar but quantitatively different. Based on these findings, the differences in the host immune response might explain a part of the difference observed in the viral pathogenesis of high and low pathogenic influenza strains in ducks.

Keywords: ducks; avian influenza virus infection; differential host immune response; microarray

Introduction

Influenza A viruses are divided into different subtypes on the basis of the surface viral glycoproteins hemagglutinin (HA) and neuraminidase (NA). Currently, 18 known HA and 11 known NA subtypes were reported (CDC, 2015). Of these, 16 HA [H1–H16] and 9 NA [N1–N9] subtypes have been isolated from wild aquatic birds (Webster *et al*., 1992; Olsen *et al.,* 2006; Krauss and Webster, 2010; Marchenko *et al.,* 2012). Influenza A virus infections in wild aquatic birds are predominantly maintained by asymptomatic condition (Taubenberger and Kash, 2010; França and Brown, 2014). Generally, ducks are considered a naturally resistant host for H5N1 influenza virus infection (Kida *et al*., 1980; Barber *et al*., 2010) and act as main reservoir for influenza A viruses (Hulse-Post *et al*., 2005; Songserm *et al.,* 2006; Kim *et al.,* 2009). Most H5N1 virus infections in ducks cause no or mild clinical disease and lesions (Alexander *et al.,* 1986; Perkins and Swayne, 2002; Songserm *et al.,* 2006). However, this situation has changed after evolution of Eurasian-African lineage of highly pathogenic avian influenza

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Abbreviations: AI(V) = avian influenza (virus); dpi = days post infection; HP = highly pathogenic; IFN = interferon; LP = low pathogenic

(AI) (HPAIV) H5N1 viruses from A/goose/Guandong/96(Gs/GD) virus. This Eurasian-African lineage of H5N1 HPAIVs causes diseases in various wild aquatic and terrestrial bird species including ducks (Ellis *et al.,* 2004; Chen *et al.,* 2005; Brown *et al.,* 2006, 2008; Pantin-Jackwood and Swayne, 2007; Pasick *et al.,* 2007; Kim *et al.,* 2012).

Various field outbreaks and experimental infection studies showed that ducks were susceptible to the infection by some Eurasian-African lineage H5N1 viruses, produce clinical signs ranging from mild clinical signs to death, with mortality rate approaching 31–80% (Sturm-Ramirez *et al.,* 2004; Zhou *et al.,* 2006; Sun *et al.*, 2011; Choi *et al.,* 2013; Haider *et al.,* 2015). The H5N1 virus-infected ducks showed the clinical signs, including torticollis, incoordination, tremors, seizures, whitish watery diarrhea, loss of appetite and sudden death (Ellis *et al.,* 2004; Sturm-Ramirez *et al.,* 2004; Kishida *et al.,* 2005; Vascellari *et al.,* 2007; Haider *et al.,* 2015).

India has been experiencing outbreaks of HPAI H5N1 virus every year since it was first reported in Maharashtra State in February 2006 (Pattnaik *et al*., 2006; Tosh *et al*., 2007, 2011; Murugkar *et al*., 2008; Nagarajan *et al.,* 2009). A/duck/Tripura/103597/2008 virus (hereafter mentioned as low pathogenic [LP] virus) was isolated from ducks and classified as H5N1 subtype and further phylogenetic analyses indicated that this virus belongs to clade 2.2, which includes viruses causing sporadic mortality in ducks. A/duck/India/02CA10/2011 virus (hereafter mentioned as highly pathogenic [HP] virus) was isolated from the natural outbreak of disease in parent duck flocks of Khaki Campbell breed in State Duck Breeding Farm, Agartala District of Tripura State. HP virus caused 61% mortality in ducks, was classified as H5N1 subtype and this virus belongs to clade 2.3.2.1 (Nagarajan *et al.,* 2012).

The viral pathogenesis of AIV is a polygenic trait, which is associated with various factors including virus strain, species, age at infection, immune status and immune responses of the host. The extensive studies in human, animal models and various *in vitro* systems clearly indicate that host innate immune response plays a critical role in viral pathogenesis and outcome of an influenza virus infection (La Gruta *et al.,* 2007; Maines *et al.,* 2008). The complete understanding of host immune responses and role of host immune response in viral pathogenesis of ducks infected with different pathotypes of H5N1 influenza virus is still poorly understood. In order to determine the role the host immune response against two differentially pathogenic virus strains infection in ducks, we studied global lung tissue immune response of ducks infected with the high pathogenic virus (HP virus) and low pathogenic virus (LP virus). Our results suggest that the differential regulation of the host immune response might in part explain the difference observed in the viral pathogenesis of high and low pathogenic influenza strain infection in ducks.

Materials and Methods

Ethics statement. The animal experiments were carried out at the Biosafety level 3+ containment facility of ICAR-National Institute of High Security Animal Diseases, Bhopal, India, as per the guidelines of Institutional Animal Ethics Committee and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forests, Govt. of India (Approval No. 68/IAEC/HSADL/12 dated 11.05.2012).

Virus. Two different H5N1 viruses isolated from ducks namely, A/duck/Tripura/103597/2008 belonging to clade 2.2 (LP virus) and A/duck/India/02CA10/2011 belonging to clade 2.3.2 (HP virus) were used in this study to assess the differential host immune response to different pathotypes. The stock viruses were propagated in the allantoic cavities of 12-day-old embryonated duck eggs. The Custom Duck 8x60k microarray (AMADID G4102A_059612) was designed using sequences available in the NCBI database for *Anas platyrhynchos* species on Agilent platform from Genotypic Technology Pvt. Ltd. A total of 23069 duck sequences were incorporated in the duck microarray chip.

Experimental infection of ducks. Six-week-old AIV-seronegative domestic ducks were divided into three groups, with each group containing 6 birds. Group 1 was intranasally inoculated with 10^6 EID_{50} of A/duck/Tripura/103597/2008 H5N1 (LP) virus isolate and the group 2 was inoculated with 10^6 EID_{50} of A/duck/India/02CA10/2011 H5N1 (HP) virus isolate. Group 3 was inoculated with PBS. Birds were observed daily for clinical signs. Three birds from each group were sacrificed at 5 days of post infection (dpi) and lung tissues collected in RNA later reagent (Ambion™) and stored at -80°C. The remaining birds were observed for clinical signs up to 7 dpi. The virus infection in lungs was confirmed by tissue inoculation in embryonated chicken eggs and hemagglutination (HA) assay.

Total RNA isolation and microarray hybridization. Total RNA was isolated from the lung tissues of three birds from each infected group and two birds from non-infected group. Total RNA was isolated using TRIzol® Reagent (Invitrogen, USA) with the Qiagen's RNeasy mini kit (Qiagen, Germany). The integrity of RNA was analyzed on the Bioanalyzer (Agilent 2100). Total RNA was labeled using Agilent Quick Amp labeling kit by standard procedure. cRNA was purified using Qiagen RNeasy column. Concentration and amount of dye incorporated into labeled cRNA was determined using Nanodrop ND-1000 UV-VIS spectrophotometer. Samples that passed the QC for specific activity were taken for hybridization. 600 ng of labelled cRNA were hybridized on the specific arrays using the Gene Expression Hybridization kit in Sure Hybridization chambers at 65°C for 16 h. Hybridized slides were washed using Agilent Gene Expression wash buffers. Washed microarray slides were scanned on a GS600D scanner (Agilent Technologies).

Microarray data analysis. Data extraction from images was done using Feature Extraction software version 10.7. Spot intensity was determined using a local background subtraction method. Percentile shift normalization method was used for normalization, where the locations of all the spot intensities in an array were adjusted. The

Table 1. Oligonucleotide primers used for validation of microarray data

Gene name	Sequence (5′-3′)	Reference / Acc. No.
Duck-MHCI-1-F	GAAGGAAGAGACTTCATTGCCTTGG	Maughan *et al.*, 2013
Duck-MHCI-1-R	CTCTCCTCTCCAGTACGTCCTTCC	
Duck-MHCII-1-F	CCACCTTTACCAGCTTCGAG	Maughan *et al.*, 2013
Duck-MHCII-1-R	CCGTTCTTCATCCAGGTGAT	
Duck-SOCS3-F	AAGACGTTCAGCTCCAAGA	XM_005031814.1
Duck-SOCS3-R	AGTAGAAGCCGCTCTCCT	

normalized raw data were then subjected to a statistical analysis using GeneSpring GX 12.5 software (Agilent) to identify differentially expressed genes. The normalized raw data results obtained with LP or HP virus-infected lungs were compared to those obtained with control. Gene expression ratios compared with the reference control were calculated and transformed to log2 base. All the expression fold change values represented in the manuscript are log2-transformed values. The cut-off of p-value ≤0.05 and fold change value +/- ≥1 (log2-transformed value) was used to identify the significant differential expression. The microarray data have been deposited in NCBI Gene Expression Omnibus (Acc. No. GSE65230). The DE gene lists of these two viruses were compared in Venny tool to identify commonly up- or down-regulated or uniquely expressed to each virus. Functional classification of the DE genes was performed for gene ontology (GO) in Database for Annotation, Visualization and Integrated Discovery (DAVID) (Huang da *et al.,* 2009) and pathway analysis in Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa and Goto, 2000).

RT-qPCR assays. The differential expression data was validated by RT-qPCR. Total RNA was isolated from triplicate of lung tissues using TRIzol ® Reagent (Invitrogen, USA) with the Qiagen's RNeasy minikit (Qiagen, Germany). cDNA was synthesized from mRNA with random hexamer primer using Revert Aid First Strand cDNA Synthesis Kit #K1621 (Thermo Scientific, USA). cDNA was subjected to real-time PCR using gene-specific primers by SYBR green chemistry. Primers used for RT qPCR were those previously reported (Maughan *et al.,* 2013) or designed using Oligo Architect tool (Table 1). RT-qPCR was done on LightCycler® 480 System Real-Time PCR System (Roche Applied Science, USA) using USB® VeriQuest® SYBR® Green qPCR Master Mix (2X) with Fluorescein (p/n 75665) (Affymetrix, Inc USA). The data obtained from the RT-qPCR was analyzed by Schmittegen and Livak (2008) method. The data was normalized using β-actin as the internal control gene. The ΔΔCt value was calculated as difference in normalized Ct value (ΔCt) from infected samples to the ΔCt from non-infected samples. The ΔΔCt value is transformed into $2^{-\Delta\Delta Ct}$ value as the estimated gene expression fold change value.

Results

Host immune gene response to low pathogenic virus infection

The LP virus-infected ducks showed clinical signs including depression, loss of appetite and watery diarrhea. Hemagglutinin titer of the LP virus was found to be 2^4. Global gene expression profiling of duck lungs infected with LP was done by microarray analysis of total RNA extracted from lung tissue collected at 5 dpi. A total of 1191 genes were significantly up-regulated and 1051 genes were significantly down-regulated (p-value ≤0.05 and fold change value +/- ≥1) (Table 2) with respect to control. We identified several immune genes up-regulated in lung tissues of duck in response to LP virus infection including 2',5'-oligo adenylate synthetase (*OAS*), MX dynamin like GTPase 1 (*MX*), suppressor of cytokine signaling 3 (*SOCS3*), interleukin 17 (*IL17*), interferon-induced protein with tetratricopeptide repeats 5 (*IFIT5*), interferon-induced transmembrane protein 5 (*IFITM5*), radical S-adenosyl methionine domain containing 2 (*RSAD2*), tripartite Motif Containing 23 (*TRIM23*), eukaryotic translation initiation factor 2 alpha kinase 2 (*EIF2AK2*), etc. Another set of the immune genes was also found to be down-regulated in LP virus infection, including toll like receptor 4 (*TLR4*), interferon gamma (*IFN-γ*), interferon alpha (*IFN-α*), and beta (*IFN-β*), receptor subunit 1 (*IFNLR1*), signal transducer and activator of transcription 4 (*STAT4*), chemokine (C-C motif) ligand 5 (*CCL5*), chem-

Table 2. Summary of differentially expressed genes in response to infection with LP and HP viruses

Infection condition	Genes qualifying the quality criteria	Differentially expressed genes (+/- ≥1 folds, p <0.05)	Up-regulated genes	Down-regulated genes
A/duck/Tripura/103597/2008 (LP virus)	7350	2242	1191	1051
A/duck/India/02CA10/2011 (HP virus)	6266	1566	770	796

Table 3. Significantly enriched gene ontology terms in response to LP and HP virus infections

GO term	HP virus		LP virus	
	Gene count	p-value	Gene count	p-value
Cellular homeostasis	38	1.60E-03	44	3.60E-02
Cytokine receptor activity	8	1.40E-02	–	NE
Immune response	45	3.00E-02	–	NE
Induction of apoptosis	23	5.50E-02	–	NE
Inflammatory response	27	7.00E-03	33	3.00E-02
Positive regulation of cellular biosynthetic process	50	2.90E-03	62	3.60E-02
Positive regulation of gene expression	44	2.80E-03	0	0.00E+00
Positive regulation of mononuclear cell proliferation	9	4.80E-03	8	9.30E-02
Regulation of apoptosis	69	2.50E-06	76	5.50E-03
Regulation of B cell activation	9	2.60E-03	8	6.20E-02
Regulation of leukocyte activation	13	9.90E-02	8	9.30E-02
Anti-apoptosis	20	4.50E-03	24	1.70E-02
Positive regulation of lymphocyte proliferation	9	4.30E-03	8	8.70E-02
Regulation of phosphorylation	34	1.50E-02	50	2.50E-03
Response to cytokine stimulus	10	1.30E-02	11	4.70E-02
Response to wounding	40	4.70E-03	55	3.10E-03
T cell receptor signaling pathway	4	9.40E-02	–	NE
Transforming growth factor beta receptor signaling pathway	9	7.30E-03	–	NE
Regulation of chemokine production	–	NE	4	8.20E-02

NE = the GO term was not enriched to a specific infection condition.

Table 4. List of genes differentially expressed in response to LP and HP virus infections

Gene name	Fold change in HP virus infection (log2 value)	Fold change in LP virus infection (log2 value)
Up-regulated in HP virus infection and down-regulated in LP virus infection		
ADAMTS1	1.002	-1.054
FAM84B	1.321	-1.231
IFN-γ	1.270	-1.275
KIAA0146	2.363	-1.320
LOC101789899	1.005	-2.568
LOC101791499	1.234	-3.831
LOC101801272	1.883	-1.702
LOC101801942	1.418	-2.869
LOC101802795	1.961	-1.230
LOC101804060	2.043	-1.521
PRKDC	2.320	-5.923
RNASEL	1.007	-1.002
SHROOM1	1.013	-1.198
SSTR1	2.614	-1.048
TAP2	2.464	-2.188
TRNAR-UCU	3.303	-2.166
Down-regulated in HP virus infection and up-regulated in LP virus infection		
CCDC37	-2.345	1.294
COLEC10	-1.474	1.447
FAM53A	-1.732	1.063
GRIP1	-1.863	1.129
ITGB6	-2.364	1.800
KLB	-2.119	1.595
LOC101790907	-2.073	1.574
LOC101791514	-2.147	2.310

Table 4. (continue)

Gene name	Fold change in HP virus infection (log2 value)	Fold change in LP virus infection (log2 value)
Down-regulated in HP virus infection and up-regulated in LP virus infection		
LOC101791947	-6.017	1.360
LOC101798529	-1.596	1.273
LOC101804087	-1.195	1.284
LRRC23	-2.362	1.905
MMACHC	-2.112	2.402
NDUFAF4	-1.935	1.385
PP2D1	-3.164	2.163
PRDX1	-2.464	1.893
RAB36	-3.105	1.268
SLC12A3	-1.263	1.040
SNTN	-2.720	1.571
UBR3	-3.202	1.248
WDR93	-2.276	1.234
YBX1	-2.504	1.837
ZMYND12	-1.364	1.076

okine (C-X-C motif) ligand 14 (*CXCL14*), *IL16*, *TRIM63*, etc. Gene ontology analysis of DE genes in DAVID revealed that the genes were involved in cellular homeostasis, B cell activation, leukocyte activation, negative regulation of apoptosis, positive regulation of molecular function, positive regulation of chemokine biosynthetic process, regulation of cytokine biosynthetic process, response to cytokine stimulus, etc. (Table 3). KEGG pathway analysis enriched the RIG-I-like receptor signaling pathway, chemokine signaling pathway, Jak-STAT signaling pathway, TNF signaling pathway, metabolic pathways, MAPK signaling pathway, cytokine-cytokine receptor interaction, TGF-beta signaling pathway, etc. (Fig. 1).

Host immune gene response to highly pathogenic virus infection

The HP virus-infected ducks gradually developed clinical signs such as depression, loss of appetite, watery eye discharge and torticollis observed on 7th dpi. Hemagglutinin titer of the HP virus isolated from infected lung tissues was 2^5. To identify genes contributing to the immune response to HP H5N1 influenza infection, we compared the infected tissue samples to non-infected samples. In HP virus infection, 770 genes were up-regulated and 796 genes were down-regulated in the lung tissues (Table 2). Among these up- and down-regulated genes, 880 genes were expressed only in HP virus infection. Particularly, HP virus induces expression of important immune genes such as *IFITM2*, *STAT3*, *CCL4* and *CXCR4* in lung tissue; these genes were reported to be associated with high pathogenic influenza virus infection in ducks. Higher induction of *IFITM* gene family, including *IFITM1*, *2* and *3* genes, during high pathogenic H5N1 virus infection in ducks determines the outcome of the disease (Smith *et al.*, 2015). Another study reported that differential expression pattern of *STAT 3* gene may also determine differential outcome of highly pathogenic virus infection in ducks (Kuchipudi *et al.*, 2014). Further KEGG pathways analysis revealed that HP virus activated various immune pathways, namely TLR, RIG-I and type I IFN pathways (Fig. 1) and results in a more pronounced induction of IFNs (IFN-γ), pro-inflammatory cytokines (IL17) and chemokines (CCL4 and CXCR4) expressed exclusively in HP virus infection conditions. The GO analysis of these differentially expressed genes in HP virus infection enriched similar biological processes as in response to LP virus infection (Table 3) (Fig. 1). However, there was quantitative difference in number of genes involved in particular biological process that were observed between the expression profiles of the two virus infections.

Comparative analysis of host gene expression responses between low pathogenic and highly pathogenic virus infection

To analyze the commonality of the host immune response to the two H5N1 viruses, LP and HP virus infections, we compared differentially expressed (up- or down-regulated) genes from each infection condition and identified the union of these gene lists. A total of 686 genes were found to be common between the two virus infections, indicating that ducks express the same 686 genes regardless of the H5N1 virus isolate (Fig. 2a). Of them, 348 genes were commonly up-regulated and 299 genes were commonly down-regulated in response to both virus infections (Fig. 2b). However, 39 genes were found to have differential expression pattern, of these 16 genes were up-regulated in HP virus infection but down-regulated in LP virus infection, and 23 genes were down-regulated in HP virus infection but up-regulated in LP virus infection (Fig. 2b) (Table 4). The functional annota-

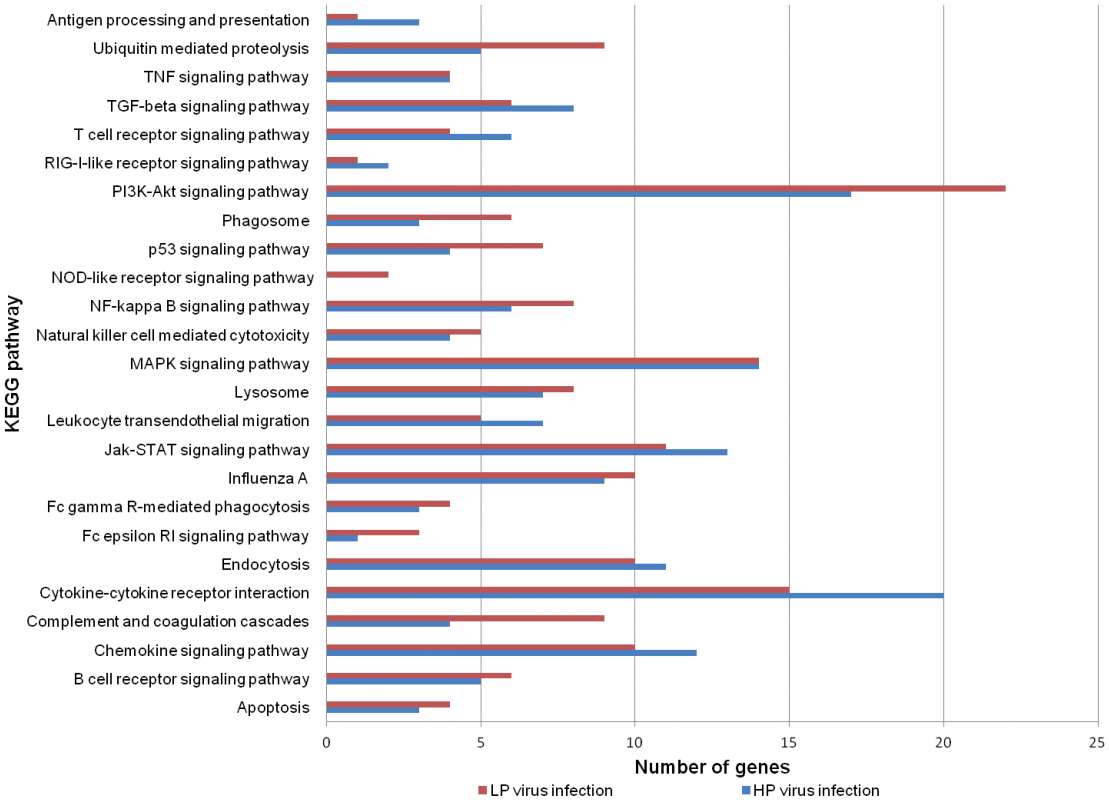

Fig. 1

KEGG pathways were differentially enriched in response to LP and HP virus infections

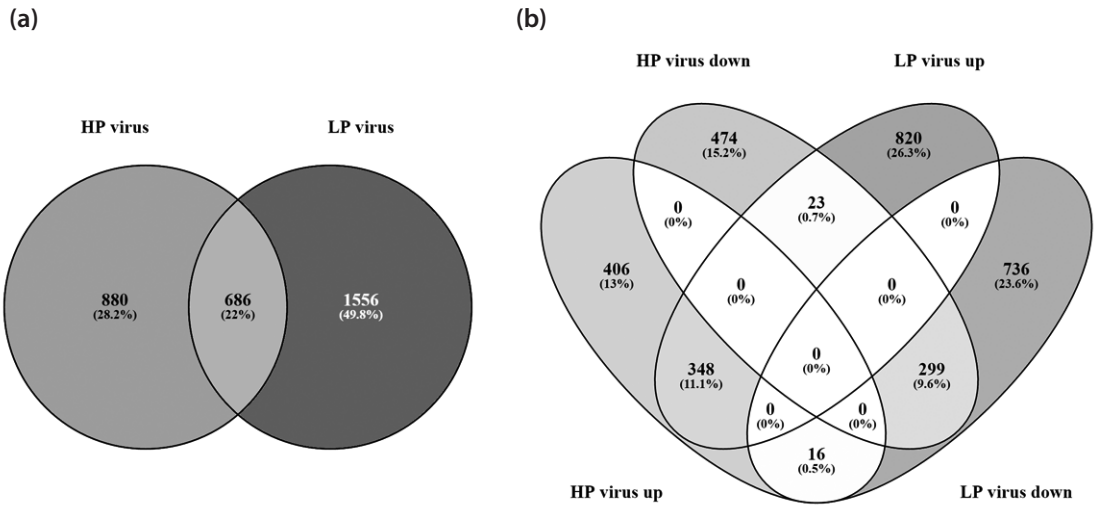

Fig. 2

Comparative analysis of gene expression changes between HP and LP virus infection conditions at 5 dpi

(a) Venn-diagram showing the comparison of genes list between HP and LP virus infection condition. **(b)**Venn-diagram showing the differential expression of commonly expressed genes between HP and LP virus infection conditions.

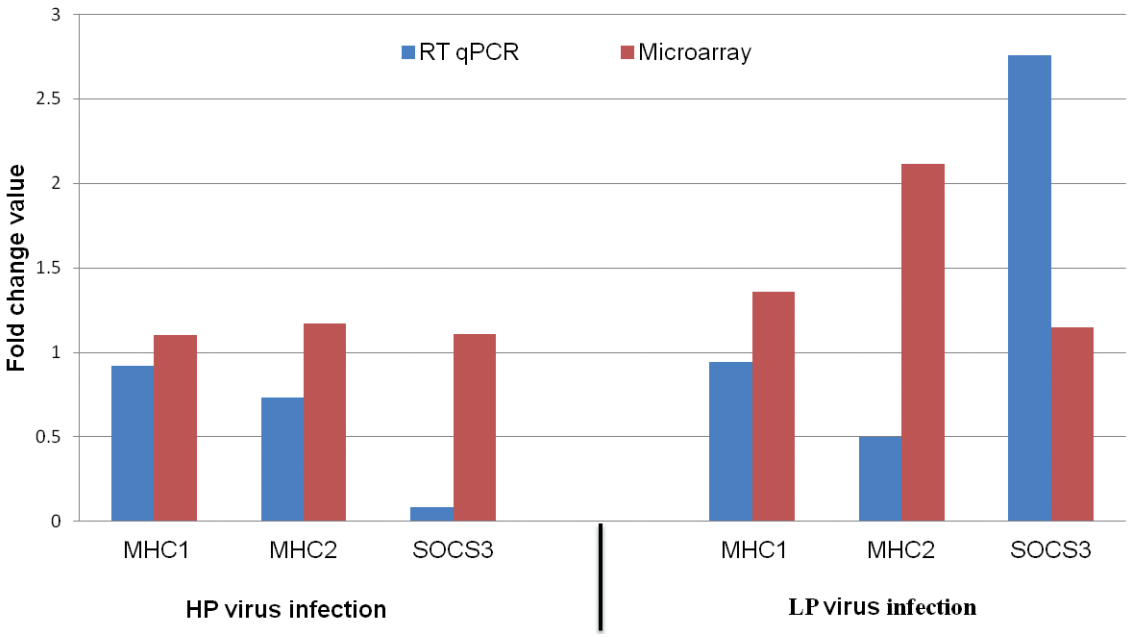

Fig. 3

Validation of microarray data by RT qPCR

tion of these 16 up-regulated genes related to the influenza A pathway, Cytokine-cytokine receptor interaction, Jak-STAT signaling pathway, TGF-beta signaling pathway and the biological process of phosphorylation and regulation of cell proliferation. Further, the functional GO term annotation of the 23 down-regulated genes related to the host cell molecular function and cellular component. This functional annotation of differentially expressed genes results indicates that host immune related pathways and biological process were strongly activated during HP virus infection and normal host cell molecular function and hemostasis were suppressed or *vise versa* in case of LP virus infection condition. In further analysis of differences in host response to HP virus compared with LP virus infection, the immune genes such as *MX*, *IFIT5*, *IFITM5*, *RSAD2*, *EIF2AK2* (PKR), β-defensins, *CXCL14*, etc. were expressed in LP virus infection and these genes were not significantly expressed in HP virus-infected lung tissues. Similarly some of the immune genes (*CCL4*, *CXCR4*, *IFITM2*, transforming growth factor beta 1 (*TGFB1*) and *TGFB3*, *IRF1*, *STAT3*, etc.) were expressed only in HP virus infections but not in low pathogenic virus-infected lung tissues.

Validation of microarray data by RT-qPCR

A set of duck immune genes was assessed for the differential gene expression in lung tissues after HP or LP virus infection by RT-qPCR. Fig. 3 illustrates the changes in gene expression of the major histocompatibility complex (*MHCs*), and *SOCS3*. These genes were selected for their known role in the response to AIV infection. In comparison to microarray gene expression data, most of the fold change values were found to be compliant with microarray data though the magnitude sometimes differed.

Discussion

At field conditions A/duck/India/02CA10/2011 (HP virus) H5N1 virus (clade 2.3.2) caused 61% mortality in ducks (Nagarajan *et al.,* 2012), whereas sporadic mortality was observed in A/duck/Tripura/103597/2008 (LP virus) H5N1 virus (clade 2.2) infection. In experimental infection condition, HP virus-infected ducks developed neurological symptoms at 7 dpi and no such symptoms or other clinical symptoms were observed at 7 dpi in LP virus-infected birds. Virus-induced host immune response plays an important role in the differences in viral pathogenesis observed between different pathotypes of influenza virus (Adams *et al.,* 2009; Cui *et al.,* 2014). In order to understand the differential host immune response, we studied the genome-wide host gene expression in duck lung tissues infected with high or low pathogenic strains of H5N1 viruses using custom-designed microarray chip.

The recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) triggers the activation of transcription factors and the expression of interferons, proinflammatory cytokines and chemokines (Kawai and Akira, 2010; Matsumiya and Stafforini, 2010; Iwasaki and Pillai, 2014). The microarray gene expression data indicate an up-regulation of *IFN-γ* in HP virus infection compared to its down-regulation in LP virus infection. *IFN-γ* up-regulation has been demonstrated in ducks in response to a LPAIV infection (Adams *et al.,* 2009; Maughan *et al.,* 2013). The H5N1 infection results in high transcriptional induction of IFNs, cytokines and chemokines in affected lung tissue and these play a major role in pathogenesis of H5N1 viruses (Baskin *et al.,* 2009). By comparative analysis we found that the pattern of host immune gene response was quite different between HP and LP virus isolate. We observed the up-regulation of *IFITM2*, *TGFB1* and *TGFB3*, *STAT3*, *CCL4*, *CXCR4* genes following HP virus infection, but not LP virus infection. Further, *OAS*, *IL17*, and *SOCS3* genes were expressed in both infection conditions, however, moderate differences were observed in expression levels of these genes in these two conditions. Previous studies have been done to compare the host immune responses of ducks infected with different pathogenic influenza strains. These studies suggest that the increased pathogenicity of more virulent influenza strain in ducks may be associated with rapid replication of the virus, accompanied by the robust host immune responses (retinoic acid-inducible gene 1 [*RIG-I*], *IFN-α*, *IL-6*, *IL-8*, *ISG12-2*, *IFIT5*, *OASL* and *IFITM1*), but minimal immune responses to a low virulent strain (Cagle *et al.,* 2011; Pantin-Jackwood *et al*., 2012; Vanderven *et al*., 2012; Wei *et al.,* 2013; Cui *et al.,* 2014).

It is noteworthy that the expression of important antiviral immune genes, including *IFIT5*, *IFITM5*, β-defensins 6, interferon-stimulated gene12-1 (*ISG12-1*), *RSAD2*, *EIF2AK2* (*PKR*), *CCL5*, *TRIM23*, solute carrier family 16 member 3 (*SLC16A3*), was observed in LP virus infection, but not in HP virus infection. β-defensins are induced in response to influenza virus infection (Chong *et al.,* 2008; Huang *et al.,* 2013), inhibit AIV replication and increase the uptake of these viruses by neutrophils (Doss *et al*., 2009). *ISG12* is involved in apoptosis of H5N1-influenza infected duck cells, which has been suggested as a mechanism of viral resistance (Kuchipudi *et al*., 2009). *IFITM5* and *IFIT* genes have key roles in the antiviral response to AIV infection in mammals as well as ducks (Barber *et al.,* 2010; Pichlmair *et al*., 2011; Huang *et al.,* 2013). The differential expression of these important antiviral immune genes may modulate the viral pathogenesis in the LP virus infection making it apparently less pathogenic in ducks.

The genes involved in antigen processing and presentation, including MHC I alpha chain, MHC II beta chain, immunoglobulin lambda constant 1 and 2 (*IGLC1 and 2*) and beta 2 microglobulin, were up-regulated in lung tissues in response to both virus infections. The up-regulation of MHC I and MHC II genes was validated by RT qPCR assay. The MHC molecules are involved in the activation of specific acquired immunity to AIV infection and eliminate the pathogen; their up-regulation during AIV infection has been reported in ducks (Vanderven *et al.,* 2012; Huang *et al.,* 2013; Wei *et al.,* 2013).

Differences in enrichment of different signaling pathways and GO terms were implicated into the difference between low pathogenic and high pathogenic influenza strain infection in ducks (Massin *et al.,* 2013; Maughan *et al.,* 2013). The cellular immune pathways such as chemokine signaling pathway, cytokine-cytokine receptor interaction, MAPK signaling pathway, NF-kappa B signaling pathway, TGF-beta signaling pathway, Toll-like receptor signaling pathway, RIG-I-like receptor signaling pathway, Jak-STAT signaling pathway, TNF signaling pathway, etc. are activated in response to infection with either virus in ducks. These pathways confirmed a similar innate immune response to both viruses; however, there were quantitative differences in number of genes involved in these pathways following infection with the two studied viruses. Further details of molecular mechanisms of activation of these pathways and interaction of these various pathways need to be studied in ducks. GO terms analysis of genes responsive to HP virus infection enriched the terms such as response to cytokine stimulus, induction of apoptosis, inflammatory response and response to wounding; these GO terms may suggest that HP virus induces hyper-immune responses in ducks. In contrast, in response to LP virus infection the terms related to regulation of apoptosis, homeostatic process, regulation of chemokine production and regulation of cytokine biosynthetic process are enriched, which may suggest that LP virus-induced successful moderate immune response allows the host to survive the LP infection.

Some of the well known AI responsive genes such as *IFN-α*, *IFN-β*, *RIG I*, *TLR3*, *TLR7*, other interleukins, etc. were not significantly recovered in both samples at cut-off of p-value ≤0.05 and fold change value +/- ≥1. This may be due to the fact that the stringent p-value cutoffs typically applied during microarray analysis can often obscure interesting trends in the expression data (Lee *et al.,* 2009). Also, the duck genome is only preliminarily annotated at present and probably, since the array was designed on the basis of this draft genome, it resulted in high background levels owing to cross-hybridization, saturation and spot density. The status of the expression of these important genes may need to be studied with a more sensitive tool like RNA seq.

In conclusion, transcriptome analysis of duck lungs infected with the different H5N1 isolates (HP and LP) reveals differences in the magnitude of host immune responses. This can be speculated to be the cause of difference in outcome of the disease

in ducks when infected with these isolates. This study provides a useful background information regarding relationship between H5N1 viral pathogenesis and host immune response in ducks. Further studies will be required to characterize the pathogenicity factors of influenza strain and the host immune response in order to understand the complete viral molecular pathogenesis and outcome of disease in ducks.

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