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Meta-analysis of RNA Seq Datasets in Duck Lungs Infected with Highly Pathogenic Avian Influenza Viruses

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ABSTRACT

The molecular mechanisms and hub genes identified for disease resistance in ducks against highly pathogenic avian influenza virus (HPAIV) infection show substantial variations between studies. This is mainly due to the limitations of small sample sizes owing to the huge cost of experimental infection and multiple other factors leading to the large variability of results. A meta-analysis can increase the statistical power for reliability and generalizability of previous studies to obtain more accurate results. Hence, this approach was used for identifying the molecular pathways and major hub genes responsible for disease resistance in ducks infected with HPAIVs. Meta-analysis using publicly available lung RNA seq datasets of ducks infected with HPAIVs, identified 339 differentially expressed genes (DEGs) (p-value cut-off ≤ 0.05); out of these, 214 genes were upregulated and 125 genes were downregulated. Network analysis of the DEGs suggests that infected ducks initiate a lower cytokine/chemokine response in comparison with control through the expression of anti-inflammatory effect genes (*STAT*s, *SOCSs* and *IL10*) and a persistent antiviral immune gene expression through activation of RIG-I/MAVS/IFNAR1-dependent pathways in the lung. Further, genes such as *STAT3, MYC, STAT1, MAPK11, RIPK1, SOCS3, SOCS1*, and *MYD88* were identified as the major regulators or hub genes responsible for disease pathogenesis in ducks. In conclusion, the powerful statistical meta-analysis approach led us to reveal molecular pathways and hub genes involved in the disease resistance mechanism in ducks infected with HPAIVs.

HIGHLIGHTS

- Duck initiates a lower cytokine response through expression of *STAT*s, *SOCSs* and *IL10.*
- Persistent antiviral gene expression by activation of RIG-I/MAVS/IFNAR1 pathways.
- The *STATs, SOCSs* and *MYD88* genes were main regulator genes for disease pathogenesis.

Keywords: Avian influenza virus, Molecular mechanisms, Meta-analysis, Duck, Pathogenesis

Highly pathogenic avian influenza virus (HPAIV) infection in chicken causes significant morbidity and mortality, while it is usually asymptomatic or exhibits only mild clinical signs in ducks (Liang *et al*., 2011; Cornelissen *et al*., 2013; Kuchipudi *et al*., 2014). Understanding the disease resistance mechanisms in ducks during HPAIV infection may reveal key components of immune pathways essential for both, prophylactic and therapeutic protection (Ranaware *et al*., 2016; Mishra *et al*., 2017; Evseev and

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Magor, 2019). Transcriptome analysis of ducks using microarrays/RNA sequence (RNA seq) tools have been used to identify the innate immune response to different avian influenza virus (AIV) infections (Huang *et al*., 2013; Maughan *et al*., 2013; Smith *et al*., 2015; Kumar *et al*., 2017). These transcriptomics studies revealed that ducks produce a RIG-I immune response and expression of cytokines, IFNs*, iNOS, STAT3,* and *IFITM* genes (Sarmento *et al*., 2008; Barber *et al*., 2010; Liang *et al*., 2011; Burggraaf *et al*., 2011; Kuchipudi *et al*., 2014; Smith *et al*., 2015; Kumar *et al*., 2017). The aforementioned gene expression studies have fruitfully identified the differentially expressed genes (DEGs), however the findings of these studies are not in complete agreement with each other, and only limited overlap exists for DEGs across the studies. Hence, this study was planned to utilize a meta-analysis approach to develop a robust conclusion on the disease resistance mechanism in HPAIV infection in ducks by utilizing multiple available RNA seq data.

Meta-analysis is a statistical procedure for combining data from multiple scientific studies to derive robust and reproducible results (Caldas and Vinga, 2014). Meta-analysis may develop a precise and robust conclusion that has greater statistical power after a systematic and rigorous integration of the available evidence (Tatsioni and Ioannidis, 2008). This conclusion is statistically stronger than the analysis of any single scientific study, due to increased sample numbers as well as elimination of potential biases associated with individual studies, accumulated effects and results (https://himmelfarb.gwu.edu/tutorials/studydesign101/metaanalyses.cfm).

The novel part of our study is that we performed a meta-analysis of publicly available duck lung RNA seq datasets from two independent studies (Huang *et al*., 2013; Smith *et al*., 2015). By combining the two RNA seq datasets we identified consistently DEGs with statistical significance. Further, we also performed functional annotation of these DEGs and a protein-protein interaction (PPI) network analysis of meta-analysis results to extract functional modules that may play important roles in the mechanism of disease resistance to HPAIV infection in ducks.

MATERIAL AND METHODS

Selection of eligible RNA seq expression datasets

RNA seq datasets in the NCBI Sequence Read Archive (SRA) and European Nucleotide Archive (ENA) databases were searched using the following keywords: 'ducks', and 'avian influenza infection', identifying 98 RNA seq datasets belonging to 4 studies for initial consideration. A total of 72 RNA seq datasets remained after removing duplicate datasets.

After removing 43 RNA seq datasets as irrelevant and the remaining 29 RNA seq datasets were found to be related to ducks infected with the influenza virus. All these RNA seq datasets were then indexed to verify if they met the following criteria such as high pathogenic avian influenza infection, lung tissues, and datasets containing both control and infected group samples. Finally, we selected 19 RNA seq datasets belonging to two independent studies examining differential gene expression in ducks against HPAIV infection. Huang *et al*. (2013) studied differential gene expression in duck lung tissue infected with either a highly or a weakly pathogenic H5N1 virus, namely, A/duck/Hubei/49/05, DK/49 or A/goose/Hubei/65/05, GS/65 (Huang *et al*., 2013). Smith *et al*. (2015) studied differential innate immune responses in ducks and chickens infected with low and high pathogenic AIVs in lung and ileum tissues (Smith *et al*., 2015). From this study, we selected only HPAIV infected duck lung tissues for meta-analysis. In total, 7 control and 12 HPAIV infected lung transcriptome datasets were selected for meta-analysis. Detailed descriptions of the samples and sequencing protocol followed can be found in the corresponding publications (Huang *et al*., 2013; Smith *et al*., 2015).

RNA sequence genome mapping

RNA sequence genome mapping and read count procedures were done using the GALAXY public server (https://usegalaxy.org/). The genome mapping of both control and infected RNA seq samples datasets against the duck genome (Ensembl Genome assembly: ASM874695v1) was done using hierarchical indexing for spliced alignment of transcripts (HISAT) spliced aligner (Kim et al., 2015). The SAM/BAM file generated from genome mapping and the GTF file downloaded from the Ensembl database (http://ftp.ensembl.org/pub/release-106/gtf/anas_platyrhynchos/) were used as inputs to the htseq-count tool (Anders *et al*., 2015). The resulting raw read count files were used for further meta-analysis procedures. To know gene expression levels at a particular

post infection time interval, the raw read count values are used in the Cufflinks suite of tools (Trapnell *et al*., 2010). The transcript assembly and Fragments per Kilobase of transcript per Million mapped reads (FPKM) estimation for mapped RNA seq data was done using Cufflinks software. Further Cuffmerge software was used for merging several Cufflinks assemblies followed by Cuffdiff software to find significant changes in transcript expression, splicing, promoter, etc.

Meta-analysis of RNA seq datasets

RNA seq meta-analysis was carried out using NetworkAnalyst, a visual analytic platform for comprehensive analysis of RNA seq gene expression datasets (Xia *et al*., 2015). We constructed data tables containing raw read counts with Ensembl ID in rows and samples/experiments delineated in individual columns for each study. After uploading the data sets in NetworkAnalyst, we annotated the data by converting different Ensembl IDs to Entrez IDs. For each gene, read counts were transformed to log2-counts per million followed by a verification of data integrity before proceeding to the meta-analysis steps. The common types of meta-analysis approaches are combining of p-values, combining of effect sizes (fold change), direct merging, and vote counting, with each method having its limitations and advantages (Tseng *et al*., 2012). Of these, we used the combining of effect sizes approach, because this method usually gives a more conservative result (Rau *et al*., 2014; Merleev *et al*., 2018; Vennou *et al*., 2020). The p-value $\leq$ 0.05 and fold change $\geq$ 2 or $\leq$ -2 were used to determine significant differential expression.

Functional annotation of DEGs

Genes identified to be differentially expressed in meta-analysis were further functionally annotated with annotation information available in the public domain. Initially, the DEGs were analyzed for gene ontology terms using the Database for Annotation, Visualization and Integrated Discovery (DAVID) (Huang *et al*., 2009). The pathway analysis was done using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database on the NetworkAnalyst web server (Kanehisa *et al*., 2017). The NetworkAnalyst web server was used for the construction of protein-protein interaction (PPI) networks for understanding the molecular mechanisms and identification of hub genes responsible for disease resistance in ducks (Xia *et al*., 2015). PPI network analysis produced one big subnetwork ("continent") with several smaller ones ("islands"). However, this subnetwork was still too complex to understand. Hence, this subnetwork was further broken down into smaller units (modules). The main idea of module analysis is to reduce network complexity and make it more easily interpretable. NetworkAnalyst offers heuristic and connection-first approaches to module analysis. Here, we used the connection-first approach, wherein, NetworkAnalyst software performs module detection based on the overall pattern of connectivity and the resulting modules are then evaluated on the basis of the pathway or functional enrichment analysis results. Topological measures, namely, degree centrality and betweenness centrality were used as criteria for the identification of the main hub genes. The degree centrality of a node is the number of connections it has to other nodes. The betweenness centrality is the number of shortest paths going through the node. Nodes with a higher degree centrality or betweenness centrality values are potentially important hubs genes in cellular signaling pathways (Jeong *et al*., 2001; Han *et al*., 2004; Hahn *et al*., 2005; Joy *et al*., 2005).

Gene set enrichment analysis is a cut-off-free method utilizing the entire list of meta genes for the detection of groups of genes that share common biological functions (Subramanian *et al*., 2005). Since GSEA includes all actual gene expression data, it can detect more sensitive differences than GO term analysis. GSEA was done utilizing the entire list of meta genes (12,091 genes) without applying any p-value cut-off for the detection of gene sets (Subramanian *et al*., 2005). GSEA analyses were performed by using the GSEA desktop application downloaded from GSEA downloads (http://www.gsea-msigdb.org/gsea/downloads.jsp). Here, we used both GO term and GSEA enrichment analyses as complementary to each other to support the molecular mechanisms responsible for disease resistance in ducks.

RESULTS AND DISCUSSION

In this study, through a meta-analysis approach, we identified a total of 339 genes in duck lungs (p-value cut-off $\leq$ 0.05 and fold change value $\geq$ 2 or $\leq$ -2) that were

consistently expressed differentially, with statistical significance, in response to infection with different strains of HPAIV. Out of these, 214 genes were upregulated and 125 genes were downregulated. The absolute range of gene expression level of upregulated genes was 5.85 to 2.12 fold and downregulated genes were 3.4 to 2.11 fold. A heat map of differentially expressed genes in HPAIV infected lung tissues is represented in Fig 1. Gene ontology (GO) analysis was carried out for both upregulated and downregulated genes using the DAVID software (Table 1).

Table 1: Gene ontology term analysis of all up/downregulated genes in ducks infected with HPAIVs

GO terms	Count	P-value
Upregulated genes		
Defense response to virus	18	7.10×10^{-12}
Type I interferon signaling pathway	12	1.50×10^{-10}
Apoptotic signaling pathway	4	4.80×10^{-02}
Positive regulation of tumor necrosis factor production	5	2.10×10^{-03}
NF-kappaB signaling	5	5.00×10^{-03}
Cellular response to interleukin-1	4	4.80×10^{-02}
Cytokine-mediated signaling pathway	10	2.10×10^{-05}
JAK/STAT cascade	6	3.00×10^{-05}
Cellular response to interferon-gamma	5	4.20×10^{-03}
Downregulated genes		
Viral transcription	26	1.80×10^{-31}
Ribosome	24	2.40×10^{-24}
rRNA processing	26	4.60×10^{-24}
Translation	27	1.50×10^{-23}
Poly(A) RNA binding	33	9.70×10^{-13}
Cytosol	52	3.60×10^{-10}
Focal adhesion	17	5.30×10^{-09}
Membrane	35	9.70×10^{-07}
Nucleolus	16	4.60×10^{-04}
Cell-cell adhesion	8	2.60×10^{-03}

The functional annotation of different gene sets identified by GSEA analysis revealed activation of cytokine-cytokine receptor interaction, NF-kappa B signaling, JAK/STAT signaling, Toll-like receptor signaling, TNF signaling, RIG-I like receptor signaling, and NOD-like receptor signaling pathways in the lung tissues (Fig 2).

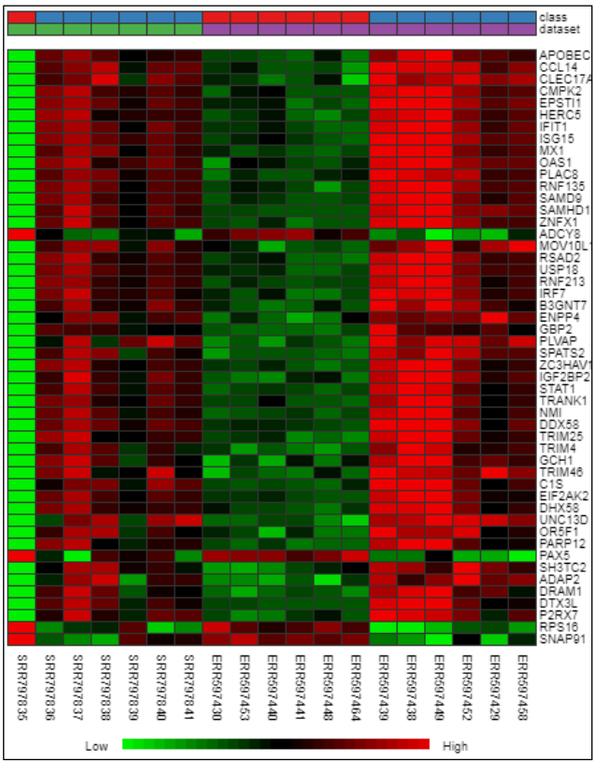

Fig 1: Heat map of differentially expressed genes of HPAIV infected lung tissues

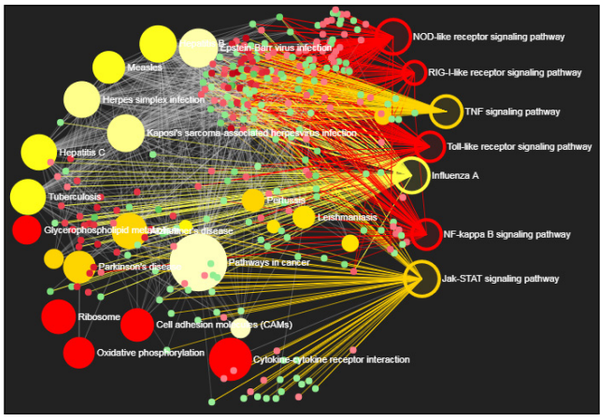

Fig. 2: Gene set enrichment analysis (GSEA) of meta-genes (12091 genes) of RNA seq datasets for detection of gene sets and functional annotation of identified gene sets

Network analysis

Disease outcome is determined by complex interactions between host and virus with altered expression and interaction of an array of host immune genes (Balasubramaniam *et al*., 2011). Among the different networks, protein-protein interactions built important networks for uncovering the molecular mechanisms of diseases.

The big subnetwork contained 1754 nodes and 4975 edges. A total of 155 genes from the meta-analysis list were mapped onto this big subnetwork. This big subnetwork was further broken down into smaller units (modules). By this method, we identified a total of 66 modules from the originally identified subnetwork. Of these, we utilized the top five modules for further functional annotation analysis (Table 2). Barber et al. have shown that ducks have an intact and functional RIG-I and the activation of RIG-I and upregulation of the genes in this pathway were observed in the duck lung tissues (Barber *et al*., 2010). Activation of NF-kappa B signaling and JAK/STAT signaling pathways were reported in the lung tissues of ducks infected with the HPAI H5N1 virus (Kumar *et al*., 2017).

Table 2: Modules identified from protein-protein interaction continent subnetwork

Module number	Size	Query	P-value	Functional annotation
1	376	36	7.82×10^{-122}	Ribosome
2	332	20	1.69×10^{-54}	JAK/STAT signaling pathway
3	48	10	8.29×10^{-08}	RIG-I like receptor signaling pathway
4	101	9	9.85×10^{-16}	NF-kappa B signaling pathway
5	108	5	3.68×10^{-31}	Regulation of actin cytoskeleton

Exhaustive molecular studies in humans and human disease model systems have acknowledged the activation of TLR signaling, RIG-I signaling, NOD like signaling, IL1R signaling, and JAK/STAT signaling pathways responsible for influenza disease pathogenesis (Teijaro *et al*., 2014). Previous literature suggests that most influenza pathogenesis causes abnormalities or modification in all of these core pathways. In our meta-analysis approach, the functional annotations of modules strongly suggest the importance of activation of RIG-I like receptor signaling, NF-kappa B signaling, and JAK/STAT signaling pathways in the lung tissues of ducks infected with HPAIV. Further, GSEA analysis of the entire list of meta-genes from the RNA seq datasets also revealed activation of RIG-I, NF-kappa B signaling, and JAK/STAT signaling pathways in lung tissues (Fig. 2). The network modules and GSEA analysis confirmed activation of RIG-I, NF-kappa B signaling, and JAK/STAT signaling pathways in duck lung tissues infected with HPAIVs at the *in-silico* level.

Further, we examined critical immune gene expression levels as a result of activation of these pathways in duck lung tissues infected with HPAIVs. The expression levels of cytokines and chemokines, such as *IL8, IL18, IL12B, IL13, IL1R1, IL6, CXCR4, CXCL12,* etc. were not reported to be significantly altered in any of the meta-analysis datasets. However, the expression level of critical antiviral immune genes such as *RIG-I, IFIH1 (MDA5), TRIM25, IRF7, IFNA, IFNG, MX1, IFITM5, OAS1, RSAD2, SOCS1, SOCS3, STAT1, IL10,* etc was highly differentially upregulated in most of these meta-analysis study data sets (Fig. 3).

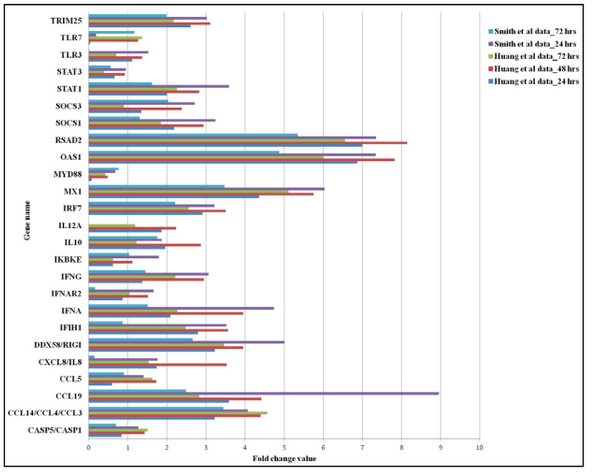

Fig 3: The expression level of critical antiviral immune genes in duck lung tissues infected with HPAIVs

Recognition of 5'ppp-RNA by the RIG-I receptor results in a conformational change that leads to exposing the caspase activation and recruitment domains (CARDs) (Pichlmair *et al*., 2006). These domains are ubiquitinated by tripartite motif 25 (TRIM25). Highly upregulated responses of

RIG-*I* and *TRIM25* were noticed in the duck lung tissues (Fig. 3). RIG-I associates with mitochondrial antiviral signaling adaptors (MAVS) to phosphorylate IRF7 and NF-kB, which further induce the expression of type I IFNs, proinflammatory cytokines, and chemokines (Loo *et al*., 2011). RIG-I is considered to be the chief regulator of the immune response during influenza infection. The presence of RIG-I in duck plays a role in clearing influenza infection, and the absence of RIG-I is speculated to be one of the factors responsible for the increased susceptibility of chicken to influenza viruses (Barber *et al*., 2010; Barber *et al*., 2013). MDA5 activates the IRF-7-dependent signaling pathway which then induces antiviral as well as proinflammatory immune responses during HPAIV H5N1 virus infection in ducks (Wei *et al*., 2014).

Typically, IFN is divided into three classes, type I (IFNA and IFNB), type II (IFNG), and type III (IFNL). Expression of antiviral cytokine IFNG during early H5N1 infection has been reported to lower viral RNA titers in H5N1 infected quail PBMC (Uno *et al*., 2013). Previous studies supported the upregulation of the IFNA gene in ducks infected with HPAIVs (Vanderven *et al*., 2012; Kumar *et al*., 2017). IFNA shows a strong antiviral activity by enhancing the downstream expression of interferon-stimulated genes (ISGs) (Qu *et al*., 2013). IFN type I and type III bind to receptor subunits, IFNAR1/ IFNAR2, and receptors IFNGR1/ IFNGR2 of the same cell or neighboring cells. When IFNs bind to the respective receptors, dimerization of the receptor subunits occurs to form the receptor complex that activates JAK/STAT signaling. The activated JAK/STAT signaling leads to the induction of a number of ISGs through ISGF3, a trimeric transcription factor. We found many ISGs (*MX1, IFITM5, OAS1, RSAD2, SOCS1, SOCS3, STAT1*) were significantly upregulated in meta-analysis data sets. Previous literature suggests the expression of several of these genes as being implicated in antiviral immunity to influenza virus infection (Turan *et al*., 2004; Silverman *et al*., 2007; Brass *et al*., 2009; Pichlmair *et al*., 2011; Smith *et al*., 2015). These findings indicate that the expression of these critical antiviral immune genes through activation of the RIG-I/ MAVS/IFNAR1-dependent pathway may restrict AIV replication and allow the ducks to recover from influenza-induced pathology.

The STAT1, STAT2, and STAT3 proteins, the key constituents of the JAK/STAT signaling pathway, play a critical role in IFN signaling and are required for a strong IFN-induced antiviral immune response (Yang *et al*., 1998; Ho *et al*., 2006). Kuchipudi et al. found that the upregulation of STAT3 gene expression blocks the inflammatory cytokine response by promoting a strong anti-inflammatory immune response (Kuchipudi *et al*., 2014). SOCS1 and SOCS3 are critical regulators of IFN-mediated immune responses and act by inhibiting the phosphorylation of STAT and inducing the expression of ISGs (Pothlichet *et al*., 2008; Pauli *et al*., 2008). The expression of IL10, an anti-inflammatory cytokine, regulates hyper induction of cytokines and chemokines during HPAIV infection in ducks. IL10 inhibits the synthesis of a number of cytokines (IFN-G, TNF, IL-3, IL-2, and GM-CSF) and enhances B cell survival, proliferation, and antibody production. Furthermore, IL10 can block NF-kappa B activity and is involved in the regulation of the JAK/STAT signaling pathway. CCL19 plays a vital role in the migration of T cells and B cells to secondary lymphoid organs and T cell trafficking in the thymus. These results indicate that the anti-inflammatory effect of STATs, SOCSs, and IL10 genes may suppress the over-abundant cytokine/chemokine production and innate inflammatory infiltrates in duck lung tissue infected with the H5N1 virus.

In summary, our meta-analysis approach suggests that infected ducks have a lower cytokine/chemokine response in comparison with control birds through the expression of anti-inflammatory effect genes (STATs, SOCSs and IL10) and a persistent successful antiviral immune gene expression through the activation of the RIG-I/ MAVS/IFNAR1-dependent pathway. These molecular mechanisms in ducks may underlie disease resistance against HPAIV infection.

Identification of hub genes responsible for disease resistance in ducks

In order to identify the main hub genes involved in the RIG-I/MAVS/IFNAR1-dependent pathway, we utilized PPI networks modules 2, 3, and 4 (Fig. 4). The genes such as *STAT3, MYC, STAT1, MAPK11, RIPK1, CDKN1A, PTK2B, SOCS3, SOCS1, BIRC2,* and *MYD88* with high degree centrality and betweenness centrality measures were identified as the main hub genes for disease resistance in ducks (Table 3).

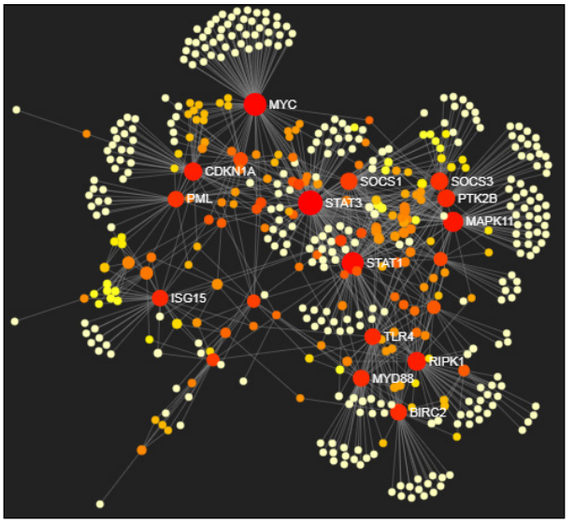

Fig 4: The RIG-I/MAVS/IFNAR1-dependent pathway of protein-protein interaction network of duck lung tissues infected with HPAIVs

The key hub genes involved in influenza molecular pathogenesis were highlighted with red color in the PPI network.

Table 3: Hub genes identified based on degree centrality and betweenness centrality measures in the PPI networks of ducks infected with HPAIVs

Sl. No.	Gene name	Degree centrality	Betweenness centrality
1	*STAT3*	113	35573.38
2	*MYC*	88	27508.61
3	*STAT1*	78	22318.25
4	*MAPK11*	58	16731.55
5	*RIPK1*	47	12785.01
6	*CDKN1A*	43	13413.57
7	*PTK2B*	39	9309.08
8	*SOCS3*	39	8026.08
9	*SOCS1*	36	4906.47
10	*BIRC2*	33	8669.15
11	*MYD88*	33	8578.55

During influenza infection, STAT3 protein has an antagonistic effect on the inflammatory cytokine response by promoting a strong anti-inflammatory response (El Kasmi *et al*., 2006; Wang *et al*., 2011; Kuchipudi *et al*., 2014; Kumar *et al*., 2017). Thus, STAT3 has a pivotal role in influenza disease pathogenesis; hence STAT3 is a probable therapeutic strategy. Currently, no molecule directly targeting the STAT3 gene/protein is available for chemotherapy (Wong *et al*., 2017). Further, small-molecule inhibitors targeting STAT3 phosphorylation or dimerization have been evaluated as antivirals *in vitro* or in animal models (Niu *et al*., 2015; Yang *et al*., 2016). STATs are essential mediators of IFN-induced JAK/STAT signaling, while the suppressors of the cytokine signaling (SOCS) family act as classical feedback inhibitors of the JAK/STAT pathway (Kershaw *et al*., 2013; Nan *et al*., 2017). SOCS members (SOCS1 and SOCS3) inhibit JAK/STAT signaling pathway by blocking the recruitment of STAT to activated cytokine receptors, binding to JAKs protein and targeting STATs and JAKs for proteasome degradation (Babon *et al*., 2012; Kershaw *et al*., 2013).

Myeloid differentiation primary response gene 88 (MyD88) is a critical adaptor protein for the TLR-dependent signaling pathways and activation of the MyD88 receptor, leading to the expression of type I interferons, inflammatory cytokines and chemokines (O'Neill *et al*., 2007). MyD88 plays a significant part in resisting primary influenza A and other respiratory virus infections (Sheahan *et al*., 2008; Seo *et al*., 2010; Leung *et al*., 2014).

p38 mitogen-activated protein kinase (MAPK11) is a member of a family of protein kinases that are involved in the integration of various biochemical signals. The previous experimental results suggest that p38 MAPK controls HPAIV-induced dysregulation of gene expression by regulating interferon synthesis and subsequent interferon signaling through a p38-specific inhibitor (Börgeling *et al*., 2014). The receptor-interacting serine/threonine protein kinase1 (RIPK1) is a member of the RIP family that acts as an important threonine/serine protein kinase in necroptosis by forming a necrosome protein complex with RIPK3 (Cho *et al*., 2009; Nogusa *et al*., 2016). In addition, both RIPK1 and RIPK3 threonine/serine protein kinases are involved in the activation of NLRP3 inflammasomes and induction of pro-inflammatory genes (Wang *et al*., 2014; Park *et al*., 2018). Further, the RIPK1 also regulates the expression of potent inflammatory cytokines, including TNF-α (Christofferson *et al*., 2018).

MYC gene expression plays a role in apoptosis, cell cycle progression, and cellular transformation (Mohamed *et al*., 2018). Further, it was reported that MYC is one of the most frequently affected genes in human cancers (Dang *et al*., 2006). In H5N1 virus infection the MYC gene has been found to be downregulated (Wang and He, 2018). The IFITM3 promoter also has a binding site for MYC transcription factors (Zhao *et al*., 2019). However, the exact role of MYC gene in influenza pathogenesis needs further investigation. Increased CDKN1A gene expression results in the production of serum amyloid A, which has a functional role in the enhanced recruitment of immune cells to the inflammatory sites (Ray *et al*., 2004). Differential expression of CDKN1A has been observed in influenza virus infection (Shoemaker *et al*., 2012; Zhai *et al*., 2015). Protein tyrosine kinase 2 beta (PTK2B) activates the MAP kinase signaling cascade through the activation of MAPK1/ERK2, MAPK3/ERK1, and MAPK8/JNK1 (Lev *et al*., 1995). Baculoviral IAP repeat-containing protein 2 (BIRC2) is a potent inhibitor of apoptosis (Salvesen *et al*., 2002). During influenza infection, BIRC2 is reported to be differentially expressed (Geiss *et al*., 2002; Pasricha *et al*., 2018). BIRC2 is required for the TNFR1 signaling pathway, nucleotide-binding and oligomerization (NOD) signaling, and RIP2 ubiquitination process (Bertrand *et al*., 2008; Bertrand *et al*., 2009). In summary, previous research strongly supports the fact that most of the identified hub genes (*STAT3, STAT1, MAPK11, RIPK1, SOCS3, SOCS1,* and *MYD88*) in our meta-analysis play important roles in the innate immune response against influenza infection and some of them may be used as novel therapeutic opportunities for the treatment of AIV infection. Further, we identified lesser-known genes (*CDKN1A, PTK2B,* and *BIRC2*) as hub genes in influenza pathogenesis.

CONCLUSION

In conclusion, this meta-analysis approach identified the expression of anti-inflammatory effect genes (*STATs, SOCSs* and *IL10*) and a persistent protective antiviral immune gene expression through activation of RIG-I/MAVS/IFNAR1-dependent pathways in HPAIV infected duck lung tissues. Further, key regulatory genes involved in the RIG-I/MAVS/IFNAR1-dependent pathway were also identified as the main hub genes responsible for influenza pathogenesis. However, the determination of critical functional roles of these genes in the disease resistance mechanism in ducks requires further biological validation by *in vitro* or *in vivo* experimentation. This meta-analysis approach consolidates the duck immune response against influenza infection and provides a proof of concept for future studies. The main limitation of this study is that the limited availability of data and more than two studies would be better for meta-analyses to understand the complete immune response in ducks infected with HPAIVs.

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