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Transcriptional response to a prime/boost vaccination of chickens with three vaccine variants based on *HA* DNA and *Pichia*-produced HA protein

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ABSTRACT

Highly pathogenic avian influenza causes severe economic losses and is a potential threat to public health. Better knowledge of the mechanisms of chicken response to the novel types of vaccines against avian influenza might be helpful in their successful implementation into poultry vaccination programs in different countries. This work presents a comprehensive analysis of gene expression response elicited in chicken spleens by a combined DNA/recombinant protein prime/boost vaccination compared to DNA/DNA and protein/protein regimens. All groups of vaccinated chickens displayed changes in spleen transcriptomes in comparison to the control group with 423, 375 and 212 identified differentially expressed genes in protein/protein, DNA/DNA and DNA/protein group, respectively. Genes with most significantly changed expression belong to immune-related categories. Depending on a group, a fraction of 15–34% of up-regulated and a fraction of 15–42% of down-regulated immune-related genes are shared by all groups. Interestingly, the most upregulated genes encode β-defensins, short peptides with antimicrobial activity and immunomodulatory functions. Microarray results were validated with RT-qPCR method, which confirmed differential regulation of the selected immune-related genes. Immune-related differentially expressed genes and metabolic pathways identified in this work are compared to the available literature data on gene expression changes in vaccinated and non-vaccinated chickens after influenza infection.

1. Introduction

Highly pathogenic avian influenza (HPAI) viruses of the H5 subtype have caused tens of millions of cases of infection in poultry (Amendola et al., 2011) and severe economic losses connected also with usage of raw infected chickens as a feed (Keawcharoen et al., 2004; Thanawongnuwech et al., 2005). Despite that only 860 human cases of avian influenza H5N1 have been reported to WHO in 2003–2017, however its mortality rate remains high (WHO, 2018). Therefore, mammalian-transmissible influenza H5N1 viruses could pose a greater threat to public health than possibly any other infectious agent currently under study in laboratories (Lipsitch and Bloom, 2012). Particularly that it is sufficient to gain mutations in 3 positions of some H5 subtypes to acquire ability for respiratory droplet transmission in ferrets (Herfst et al., 2012; Imai et al., 2012).

Chicken is an interesting research model for several reasons. It is related to mammals closely enough to have many recognizable features of the immune system and yet it is distant enough to provide an interesting basis for comparisons, with some familiar components having different characteristics e.g. chickens have far less a variety of immunoglobulins (Sun et al., 2013) and far more a variety of immunoglobulin-like receptors than mammals (Rogers et al., 2008). Moreover, several chicken genomes have been sequenced to date, which provides a sufficient amount of data for such comparisons. Finally, poultry is grown worldwide in large holdings of broilers and laying hens, as well as in small backyard flocks, and an uncontrolled burst of infection (such as avian flu) creates a risk of zoonoses for farmworkers and human populations at both local and global levels. Therefore, HPAI viruses are important poultry pathogens, not only for economic reasons but also for public health reasons, and their effective control, for example by well-developed vaccinations programs, is an important objective worldwide.

So far, poultry vaccination programs have been implemented in several countries to control the spread of both low pathogenic avian

Abbreviations: AI, avian influenza; BCRs, B cell receptors; DEGs, differentially expressed genes; HA, hemagglutinin; HI, hemagglutination inhibition; HPAI, highly pathogenic avian influenza; ImmDEGs, immune-related differentially expressed genes; PAMPs, pathogen-associated molecular patterns; TCRs, T cell receptors

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influenza (LPAI) and HPAI viruses (Eggert et al., 2010; Ibrahim et al., 2015). However, the effects of these programs are debatable, because they have not led to the eradication of the avian influenza (AI) virus in Asia and Middle East countries. The used "classical" category of inactivated vaccines does not allow for differentiation between infected and vaccinated animals (DIVA) which is the main reason for neglecting the AI vaccination programs for poultry grown in European countries. The subunit vaccines, based only on the selected antigen(s) of the pathogen, become an attractive option because they allow the DIVA monitoring strategy to be applied (Capua and Alexander, 2008; Capua et al., 2003; Wu et al., 2009). Several categories of subunit vaccines against AI, including recombinant "live inactivated vector"-based, recombinant protein-based or DNA-based, are under development ([O.I.E.] World Organization for Animal Health, 2017).

Successful vaccination depends on many factors and should cause the differentiation of memory T and B cells altogether with full response of the immune system, including such processes as: antigen recognition by pathogen-associated molecular patterns (PAMPs) receptors; signal transduction by immune-related cascades; activation of immune-related transcription factors (e.g. NF-κB) and, as a consequence, the increased production of proteins connected to exogenous antigen recognition and response, including major histocompatibility complex (MHC), immunoglobulins (Igs), T cell receptors (TCRs), cytokines, peroxidases, granzymes, lysozymes and antimicrobial peptides. Simultaneously to pro-inflammatory response, a regulatory response begins (Cohn, 2009).

Complete chicken genome microarrays provide a system-wide tool for the analysis of immune response to vaccination at the level of gene expression. Hemagglutination inhibition (HI) titre of the serum is a good predictor of influenza vaccine effectiveness; however, gene expression microarrays offer the possibility of deeper investigation into the mechanism of action, influence on the host organism and safety of the applied vaccine. Relatively few published studies have used such an approach to study vaccine immunogenicity, even in humans (Flanagan et al., 2013); however, the technology is accessible and should be more widely used in the future.

In this study, we investigated gene expression changes in the spleens of chickens immunised with three variants of subunit vaccines, based on the hemagglutinin (HA) from H5N1 virus, in comparison to the control group, which was administered an empty vector (pCI). Three vaccine regimens with prime/boost vaccination of chickens with *HA* DNA and *Pichia*-produced protein have been previously described (Stachyra et al., 2017). Although all of them (DNA/DNA, protein/protein or DNA/protein) were effective in mounting the anti-HA humoral response in chickens, the higher medians of the hemagglutination inhibition (HI) tests and the lower percentage of the competition in the influenza H5 antibody competition test in sera of animals from the DNA/DNA and DNA/protein groups suggested a slight superiority of these two vaccine variants over the protein/protein variant (Stachyra et al., 2017).

Publicly available data on gene expression differences after chicken influenza vaccination are limited. In the reported cases, birds were vaccinated with inactivated H9N2 virus (A/Chicken/United Arab Emirates/99) with or without an adjuvant and subsequently infected with homologous virus (Degen et al., 2006; Reemers et al., 2010). Therefore, to our knowledge, this is the first study reporting transcriptome changes in uninfected chickens immunised with anti-influenza vaccine.

2. Materials and methods

2.1. Spleen material and RNA isolation

The spleens were collected from the broilers immunised in previously described experiments ((Stachyra et al., 2017); Experiment 1). Briefly, a combined DNA/protein prime/boost vaccination was compared with the protein/protein and DNA/DNA approach. The K3 plasmid containing the cDNA encoding full length (except the 341-RRRKKR-347 residues) hemagglutinin from A/swan/Poland/305-135V08/2006 (H5N1) was used as DNA vaccine and *Pichia pastoris*-expressed HA (residues 17–531, with the deletion ΔRRRKKR), from the same H5N1 virus strain was used as a protein vaccine (rHA). The empty pCI vector (Promega, Poland) was used as a negative control. Here, 8-day-old chickens were primed, 22-day-old chickens were boosted and 29-day-old chickens were sacrificed. The spleens were collected one week after the booster and stored in RNA*later* solution (Ambion; 5:1; RNA*later*:tissue; v:v). Whole spleens were homogenised in Buffer RLT Plus (Qiagen; 1 g tissue/30 ml) with the ULTRA-TURRAX Tube Disperser (IKA, Germany). According to the manufacturer's protocol, 700 µl of the lysate was used for RNA isolation with RNeasy Plus Mini Kit (Qiagen). Next, despite on-column DNA digestion, all samples were additionally DNased with TURBO DNA-free™ Kit (Ambion) according to manufacturer's protocol. The DNA contamination was tested by PCR reaction with *Taq* polymerase and primers to ACTA1 intron. The RNA quality, integrity and the concentration was evaluated using Bioanalyzer 2100 (Agilent Technologies) and Nano Drop ND-1000 Spectrophotometer. RIN values were greater than 9.2, A260/280 values were greater than 1.92 and A260/230 values were greater than 0.95.

2.2. Microarray experiment

Next, 100 ng of total RNA that passed the initial quality control screen (see above) was prepared for Affymetrix whole transcriptome microarray analysis by the sequential use of the Ambion® WT Expression Kit and the Affymetrix GeneChip® WT Terminal Labelling Kit according to the manufacturer's protocols. Then, the samples were hybridized to the Chicken Gene 1.1. ST array Strips (Affymetrix, Santa Clara, CA). Microarrays were scanned with Affymetrix GeneAtlas System and the intensity of the signals for each of the probe set were written by Affymetrix software into the CEL files. The CEL files were imported into the Partek Genomic Suite v 6.15 software with the use of RMA (Robust Multiarray Averaging). The probe intensities were quantile normalised (Bolstad et al., 2003), $\log_2$ transformed and median polish summarisation to each of the probe sets was applied. Then, the qualitative analysis was performed, e.g. Principal Component Analysis, in order to identify outliers and artefacts on the microarray. After the rejection of the female chickens (based on the expression of genes localized in the W chromosome) which were identified as apparent outliers, 2, 2, 3 and 4 chickens per group remained in the negative control; DNA/DNA; DNA/protein and protein/protein group, respectively. Then, the Analysis of Variance (ANOVA) was performed on the data, which allowed lists of significantly and differentially expressed genes (DEGs) between the biological groups to be created (with the following cut-off values: p-value ≤ 0.05 and $\geq \pm 1.3$ fold change. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE102972 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE102972).

2.3. Microarray data analysis

Genes non-annotated by Affymetrix were identified with BLAST (https://blast.ncbi.nlm.nih.gov/). The obtained lists of Gene Symbols was used for functional annotation of individual genes and identification of significantly overrepresented GO terms using the Database for Annotation, Visualisation and Integrated Discovery (DAVID) v6.8 (https://david.ncifcrf.gov/) and the chicken whole genome background. Genes not annotated or unclearly annotated with DAVID were searched with other publicly available databases such as GeneCards (http://www.genecards.org), ImmGen (https://www.immgen.org/), InnateDB (http://www.innatedb.com/) and PubMed (https://www.ncbi.nlm.nih.gov/pubmed/). Venn diagrams were drawn using UGent webtool (http://bioinformatics.psb.ugent.be/webtools/Venn/) and