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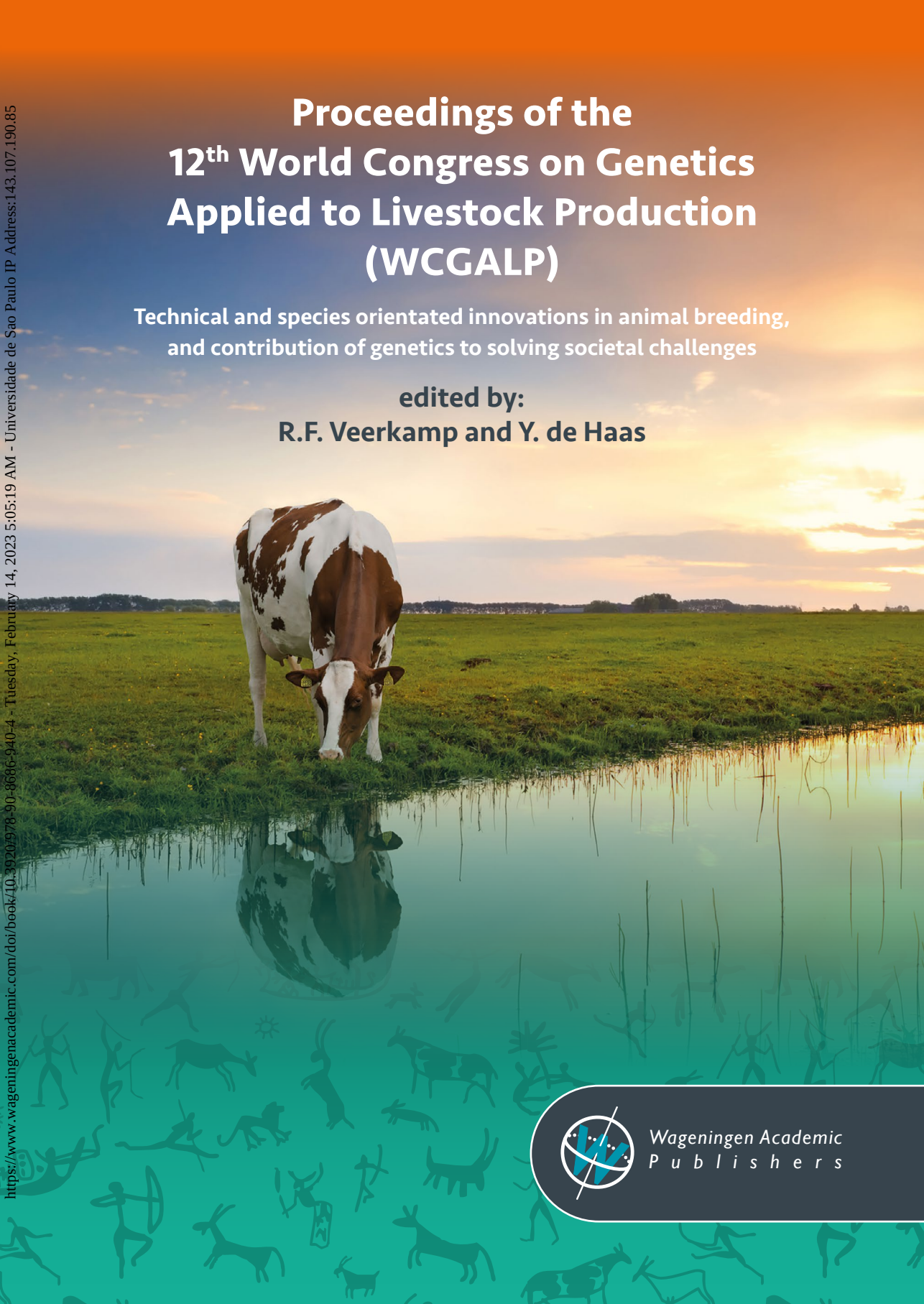

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153. Molecular mechanisms involved with influenza A nanovaccine immunogenicity in pigs

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Abstract

Influenza A virus (IAV) is one of the most important primary agents in porcine respiratory disease complex (PRDC). The gene expression profile was evaluated by RNA-Seq analysis in mediastinal lymph nodes from non-vaccinated and vaccinated pigs with a nanovaccine for IAV to clarify molecular mechanisms and genes involved in the protective immunity conferred by influenza vaccination. A total of 14,381 genes were expressed in the analysed tissue. From those, 564 were differentially expressed (DE) between the two groups. Eighteen biological processes (BP) were enriched in immune response, cell cycle regulation and reproductive processes. The defence response was the BP with more gene counts, including chemokines (*CXCL9, CXCL10, CCL8, CXCL8*), interleukin (*IL21, IL26*), interferons (*ISG20, IFN-alpha-8*), within others. The transcriptome analyses provided a better understanding of the immunogenicity in pigs induced by nanovaccination and could support the development and evaluation of better vaccines for swine and other animal species.

Introduction

Influenza A virus (IAV) is one of the most important primary agents in porcine respiratory disease complex (PRDC), causing a major economic concern for the swine industry and a pandemic threat for humans. Although the economic impact of influenza infection in pig farms is difficult to estimate, an influenza outbreak in a non-immune pig herd causes an acute respiratory disease (up to 100% morbidity), increased number of abortions and other bacterial secondary infections. In general, influenza outbreaks observed in pig farms over time increase the mortality rates (up to 2%) and cause a 10 to 30% reduction in piglet weaning weight (Gillespie *et al.* 1999; Torremorell *et al.* 2009).

In Brazil, IAV is the main virus detected in PRDC (Silva *et al.*, 2013). The genetic diversity of IAV in swine has increased since the emergence of pandemic H1N1 (pdm) virus in 2009 (Schaefer *et al.* 2015), introducing new challenges for the diagnostic and for the development of cross-protective vaccines. H1N1, H1N2 and H3N2 virus subtypes are prevalent in pig herds in Brazil, and they have distinct genetic and antigenic profiles. Hence, vaccination of swine with IAV strains that match the strains currently circulating in swine is the main strategy to control the disease in pig herds (Salvesen;Whitelaw, 2021). Although several influenza vaccines for pigs have been tested, and their efficacy proved in field trials, very few data are available on the immunological mechanisms underlying nanovaccine-induced protection. Previous study has shown a robust humoral immune response in pigs vaccinated with an influenza nanovaccine (Haach *et al.*, 2021). Therefore, we evaluated the gene expression profile in mediastinal lymph nodes (LMD) of pigs non-vaccinated and vaccinated with a nanovaccine for IAV containing the hemagglutinins of H1N1pdm, H1N2 and H3N2 viruses, to clarify the molecular mechanisms and genes involved with the immune response for a virosome-based influenza vaccine.

R.F. Veerkamp and Y. de Haas (eds)

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Materials & methods

Animals and sample collection. A total of 40 specific pathogen free (SPF) pigs were raised at the Embrapa Swine and Poultry National Research Centre, Concórdia, SC, Brazil, in a standard pig production system with water and feed *ad libitum*. The 28 days-old pigs were randomly distributed into two groups: G1: 10 non-vaccinated pigs, which received PBS injection and G2: 30 vaccinated pigs which received two doses (with 14 days of interval) of an IAV nanovaccine containing the hemagglutinins of H1N1pdm, H1N2 and H3N2. Animals were monitored for clinical signs, as well as for IAV or other virus and bacteria infection. Fifteen days after the second dose, all pigs were euthanized for mediastinal lymph node collection. LMD were stored in liquid nitrogen for gene expression analysis. The sample collection was performed according to the ethical guidelines of the Embrapa Swine and Poultry Ethics Committee on Animal Utilization, under the protocol number 001/2017.

RNA extraction, library preparation and sequencing. For the gene expression analysis, a total of 24 pigs (8 non-vaccinated, 5 females and 3 males and 16 vaccinated, 8 females and 8 males) were selected. The total RNA was extracted from approximately 100 mg of frozen mediastinal lymph node tissue using Trizol (Life Technologies), according to the manufacturer's protocol, followed by an RNA cleanup using the RNeasy mini kit (Qiagen). The RNA concentration was measured with Biodrop spectrophotometer (Biodrop) and the RNA integrity was verified using Bioanalyzer 2100 equipment (Agilent). The libraries were prepared using the Illumina Truseq Stranded mRNA (Illumina), with 1µg of total RNA, following the manufacturer's protocol. The sequencing was performed in Illumina NextSeq 2000 sequencer (lllumina, USA), using 2×100 bp paired-end reads, at the NGS Soluções Genômicas facility, in Piracicaba, São Paulo, Brazil.

Data analysis and functional annotation. The data were analysed using the Bioinformatics tools for quality control (QC) and mapping reads using BAQCOM pipeline available in the Github repository (Oliveira, 2022), which uses Trimmomatic (Bolger; Lohse; Usadel, 2014) to remove short reads (<70 bp), reads with low quality (QPhred <20) and adapter sequences, STAR (Dobin *et al.*, 2013) for mapping against the swine reference genome (Sscrofa 11.1, Ensembl release 104) and the HTseq-count (Anders *et al.*, 2015) for reads counting. The differentially expressed (DE) genes were obtained using the limma package (Ritchie *et al.*, 2015) and considered DE when a false discovery rate (FDR) was <0.05 and a logFC >|1|. Negative and positive fold-changes indicate down and upregulation of the genes in the vaccinated compared to non-vaccinated groups. The functional annotation of the DE genes was performed with clusterProfiler (Wu *et al.*, 2021) package from R language using gene ontology (GO), MSigdb and Reactome databases. Biological processes (BP) with FDR<0.05 were considered enriched. A Gene-Concept Network, which considers potentially biological complexities within genes and BP, was also constructed in the clusterProfiler.

Results

A total of 397,856,687 paired end reads were obtained for the 24 LMD samples. After QC, an average of 31.1±6.5 million paired-end reads per sample were kept, and 94.42% were uniquely mapped across the swine reference genome. Out of those, 80.63% were mapped in genes. A total of 14,381 genes were expressed in the swine mediastinal lymph node. From those, 564 were DE between groups: 316 were down and 248 were upregulated in the vaccinated group. A total of 481 genes had gene names assigned and were considered annotated, and therefore used for the enrichment analysis. After removing redundant GO terms, 18 BP were enriched in this dataset, where most of them were related to immune response, cell cycle regulation and reproductive processes. The defence response was the BP with more gene counts (25 genes), including chemokines (*CXCL9, CXCL10, CCL8, CXCL8*), interleukin (*IL21*), interferons (*ISG20, IFN-alpha-8*). In this BP, the activation induced cytidine deaminase gene (*AICDA*) gene was included,

which was the most upregulated gene in response to vaccination. Furthermore, it was possible to observe different BP that have genes with multiple functions, such as *CDC6, CDC20, CCNB1, RMI2, SGO1, TOP2A, BIRC5, ZWINT, AURKB, KIF18A, NUF2* and *HJURP*.

Using the immunologic signature of gene sets (C7) available in MSigdb, that are based on human and mouse datasets, 32 genes DE in our study have already been associated to the immune response to IAV: *MYBL2, RRM2, UBE2T, ASF1B, E2F2, CDC20, KIF2C, CDCA8, CDCA5, TOP2A, POLQ, HJURP, CENPE, BIRC5, NCAPG, KNL1, MKI67, ZWINT, GTSE1, AURKB, DLGAP5, KIF18A, E2F8, ESPL1, NCAPH, CDCA2, BUB1, CDCA7, LGI4, CKB, LPL* and *AZU1*.

Discussion

Nanovaccines can potentialize the immune response for a given antigen and could be one of the strategies to improve the performance of swine IAV vaccines (Salvesen; Whitelaw, 2021). In this study, the LMD gene expression profile in response to an IAV polyvalent nanovaccine developed by Embrapa Swine and Poultry was evaluated, evincing genes in several BPs related to the regulation of humoral immune response, immune cell migration and cell differentiation. These results corroborate with Haach *et al.* (2021), since a robust humoral immune response has already been described after vaccination of pigs with a polyvalent nanovaccine against IAV. Genes such as *AMCF-II, CXCL9, CXCL10* and *CXCL8*, known by their function in humoral response, were within the 40 genes most upregulated in the vaccinated group. The *AMCF-II*, an alveolar macrophage-derived chemotactic factor has antiviral activity (Borca *et al.*, 2021), and the *CXCL9* and *CXCL10* are activated in response to IAV in humans (Wang *et al.*, 2011).

The *AICDA* was the most upregulated (logFC=4.49) in response to the IAV nanovaccination. This gene is essential to generate high-affinity antibodies, robust humoral immunity and immune memory, and its activation in response to influenza vaccination has been observed in humans (Frasca *et al.*, 2015). It has been suggested that this gene could be a predictive marker of individual response to IAV vaccination (Frasca *et al.*, 2015). Other highly expressed genes, such as *IL26* and *IL21* are also involved in host defence and inflammatory response (Che *et al.*, 2020). Besides the functional candidate genes, which are potential markers for immune response, several genes that are important for the activation/proliferation of B and T cells were also highly expressed. Therefore, the transcriptome analyses provide a better understanding of the immunogenicity in pigs induced by nanovaccination and could support the development and evaluation of better vaccines for swine and other animal species.

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