Open Access
Asian Australas. J. Anim. Sci.
Vol. 29, No. 7 : 1044-1051 July 2016
http://dx.doi.org/10.5713/ajas.14.0923
www.ajas.info
pISSN 1011-2367 eISSN 1976-5517

Identification of Molecular Signatures from Different Vaccine Adjuvants in Chicken by Integrative Analysis of Microarray Data

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ABSTRACT: The present study compared the differential functions of two groups of adjuvants, Montanide incomplete Seppic adjuvant (ISA) series and Quil A, cholesterol, dimethyl dioctadecyl ammonium bromide, and Carbopol (QCDC) formulations, in chicken by analyzing published microarray data associated with each type of vaccine adjuvants. In the biological function analysis for differentially expressed genes altered by two different adjuvant groups, ISA series and QCDC formulations showed differential effects when chickens were immunized with a recombinant immunogenic protein of *Eimeria*. Among the biological functions, six categories were modified in both adjuvant types. However, with respect to "Response to stimulus", no biological process was modified by the two adjuvant groups at the same time. The QCDC adjuvants showed effects on the biological processes (BPs) including the innate immune response and the immune response to the external stimulus such as toxin and bacterium, while the ISA adjuvants modified the BPs to regulate cell movement and the response to stress. In pathway analysis, ISA adjuvants altered the genes involved in the functions related with cell junctions and the elimination of exogenous and endogenous macromolecules. The analysis in the present study could contribute to the development of precise adjuvants based on molecular signatures related with their immunological functions. (**Key Words:** Vaccine Adjuvant, Chicken, Reanalysis, Microarray)

INTRODUCTION

An adjuvant is an agent that stimulates the immune system and increases the host response to an antigen without itself conferring a specific antigenic effect (Bowersock and Martin, 1999). Effective adjuvants utilize multiple compounds and mechanisms to achieve the desired immunological enhancement such as long lasting antigen depots, immunological presentation of vaccine antigens, and induction of T lymphocyte responses (Reed et al., 2009). As of present, much progress has been made to develop novel adjuvants that augment humoral and cell-mediated immunity by enhancing efficacy of vaccines (Bowersock and Martin, 1999; Newman and Powell, 1995), which is particularly crucial for commercial poultry industries in tackling economically important diseases such as *Eimeria* protozoa-induced avian coccidiosis (Shirley and Lillehoj, 2012).

Examples of adjuvants used with variety of vaccines include Montanide incomplete Seppic adjuvant (ISA) series, ISA 70 VG (ISA 70) and ISA 71 VG (ISA 71), and Quil A, cholesterol, dimethyl dioctadecyl ammonium bromide, and Carbopol (QCDC) adjuvant complex, whereby the former is a water-in-oil emulsion and the latter is composed of Quil A, cholesterol, dimethyl dioctadecyl ammonium bromide (DDA), and Carbopol (Aucouturier et al., 2006; Cox et al., 2003; Dominowski et al., 2009).

ISA 70 and ISA 71 have been successfully applied to enhance immune response against pathogens of poultry, cattle, and small ruminants (Dupuis et al., 2006). Previous studies have shown that either ISA 70 or ISA 71 in conjunction with the recombinant profilin, which is an *Eimeria* specific antigen, enhances protective immunity against experimental avian coccidiosis in chicken (Jang et al., 2010; Jang et al., 2011; Lee et al., 2011). Also, other than the Montanide ISA series, improvement in vaccine responses against a variety of veterinary pathogens has been demonstrated in the use of QCDC adjuvants (Dominowski

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Submitted Dec. 8, 2014; Revised Jan. 9, 2015; Accepted Feb. 11, 2015

et al., 2009). It has been understood that Carbopol, such as dextran, polyethelyne, glycol, or polyacrylic acid that has been added to the QCDC, improves the solubility of DDA and thus makes the final formulation a highly effective adjuvant. Further incorporation of Bay R1005, a synthetic glycolipid analogue, endows the complex with the ability to stimulate both Th1-and Th2-type immunity, giving the QCDCR adjuvant a broad range of desirable immune enhancing characteristics (Dominowski et al., 2009).

Chickens infected with *Eimeria* spp. commonly develop protective immunity against reinfection by the homologous parasite, which makes immunization with parasite vaccines a viable method to control coccidiosis (Lillehoj et al., 2000a). Basically, profilin in *Eimeria* stimulates cell-mediated immunity against experimental avian coccidiosis, which is what makes it a promising vaccine candidate (Lillehoj et al., 2000b; Yarovinsky et al., 2005). In addition, the evidence of profilin shown as a potential immunogenic protein has been published in two studies that, when Montanide ISA series and QCDC formulations were used, efficacy of the profilin vaccine was improved and thus lead to more protective immunity against coccidiosis (Jang et al., 2013). However, molecular signatures related to immune-stimulatory activities of these adjuvants have not been analyzed comparatively.

Therefore, in this study, we attempted to comparatively analyze integrated microarray data from two vaccine adjuvants in experimental coccidiosis model, i.e. ISAs and QCDCs, which confer protective immunity in combination with parasitic antigen, and sought to identify common gene ontology (GO) and pathways that are targeted by these adjuvants. The idea of integrating datasets from independent, but related, sources for a comparative analysis for this study stemmed from the increasing amount of research that has used microarray technology based datasets from publicly available repositories such as Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo), ArrayExpress (http://www.ebi.ac.uk/arrayexpress), and Stanford Microarray Database (http://smd.princeton.edu).

MATERIALS AND METHODS

Associated microarray data

To investigate common immunological effects of four adjuvants, we used the Agilent Chicken Gene Expression Microarray dataset from two previous studies on vaccination effects that were obtained from GEO. In one of the studies, Montanide ISA 70 VG (ISA70) or Montanide ISA 71 VG (ISA71) with an *Eimeria* recombinant profilin protein was the experimental adjuvants used to immunize chickens subcutaneously, in comparison with only profilin immunization (accession number GSE40743). The other study used two novel adjuvant formulations, which were a combination of QCDC or QCDCR with a recombinant profilin (accession number GSE24966). The dataset used in this study was in reference to these previous studies, which contains details on immunization procedures (Kim et al., 2012; Jang et al., 2013). To briefly describe the procedure, seven-day-old Ross broiler chickens were subcutaneously immunized with profilin emulsified in each adjuvant or profilin alone. At 7 days post immunization, splenic lymphocytes were prepared from the chickens. Total RNAs were isolated from the cells using Trizol (Invitrogen, Carlsbad, CA, USA) and amplified with cyanine 3 (Cy3)- or Cy5-labeled CTP. The labeled RNAs were then hybridized to a Chicken Gene Expession Microarray (Agilent Technologies, Santa Clara, CA, USA).

Microarray image analysis was performed to analyze immunological effects of adjuvants specifically, thus the four adjuvants were categorized into two groups (Table 1). Adjuvants were grouped according to ISA adjuvants (ISA70 and ISA71) and QCDC adjuvants (QCDC and QCDCR).

Identification and functional analysis of differentially expressed genes

R package 'limma' was used to normalize and qualify microarray images. Median signal intensities were corrected by adaptive background correction (Ritchie et al., 2007) and normalized by locally-weighted scatterplot smoothing method. The log2-transformed fold changes and standard errors were estimated by fitting a linear model and empirical Bayes statistics was applied for smoothing standard errors. Differentially expressed genes (DEG) were filtered by cutoff 0.1 of false discovery rate (FDR), with adjusted p-value of two-sample t-test. Annotation of DEGs and biological function analysis were performed using DAVID Bioinformatics Resources (http://david.abcc.ncifcrf.gov/).

Table 1. Designs used in microarray image analysis using R 'limma' package

Adjuvants group	GEO series No.	Case	Control	Reference
ISA adjuvants	GSE40743	ISA70+profilin ISA71+profilin	Profilin	Jang et al. (2013)
QCDC adjuvants	GSE24966	QCDC+profilin QCDCR+profilin	Profilin	Kim et al. (2012)

ISA, incomplete Seppic adjuvant; QCDC, Quil A, cholesterol, dimethyl dioctadecyl ammonium bromide, and Carbopol.

RESULTS

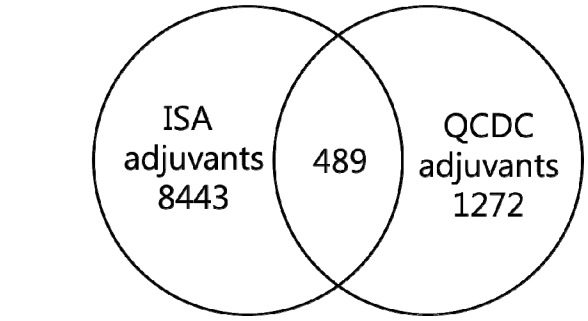
Identification and annotation of differentially expressed genes

In the present study, microarray datasets from two independent studies were combined to analyze the discriminative effects of two different types of adjuvants, Montanide ISA series and QCDC formulations. All adjuvants were administered with recombinant profilin antigens. In the results, ISA adjuvants modified greater number of genes than those of QCDC adjuvants. The numbers of DEGs were 8,932 and 1,761 in the immunization of ISA adjuvants and QCDC adjuvants, respectively. The common DEGs altered by both ISA and QCDC adjuvants were 489 (Figure 1A).

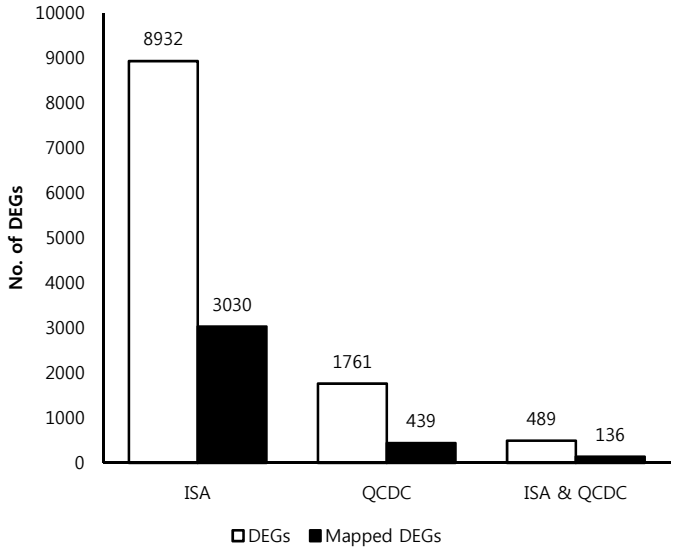
In DEGs annotation analysis, DAVID annotation analysis tool was used. Among 8,932 DEGs from the ISA adjuvants immunization, 3,030 genes (33.9%) were mapped to the chicken gene names in DAVID database. In the treatment of QCDC adjuvants, 439 (24.9%) out of 1,761 DEGs were mapped to the chicken genes in DAVID database. Among the 489 genes, which were altered commonly by both adjuvants, 136 genes (27.8%) were annotated in DAVID (Figure 1B). The annotated DEGs were used to identify their biological functions and the pathways in which the genes are involved.

Biological function analysis

In the GO analysis, 165 and 27 terms of biological process (BPs) were significantly identified from the DEGs by the treatment of ISA and QCDC adjuvants, respectively. The GO terms of BP modified by ISA or QCDC adjuvants were grouped into 23 and 8 upper categories (Table 2). Among the upper categories, six terms, i.e., "Anatomical structure development", "Cell death", "Regulation of metabolic process", "Response to stimulus", "Signal transduction", and "Single organism cellular process" were common to both adjuvant groups. The significant BPs related with the terms of "Response to stimulus" and "Cell death" were shown in Table 3 and Figure 2, respectively. All significant BPs identified were listed in Supplementary Table S1.



(A)



(B)

Figure 1. Differentially expressed genes (DEGs) and their annotation. (A) Venn diagram showing the number of DEGs by the treatment of two different types of adjuvants. False discovery rate (FDR) <0.1, (B) DEGs mapped to the chicken gene names in DAVID database.

Table 2. Categories for the significant biological processes identified from DEGs in the treatment of two different adjuvant groups

ISA adjuvants	QCDC adjuvants
Anatomical structure development	Anatomical structure development
Cell death	Cell death
Regulation of metabolic process	Regulation of metabolic process
Response to stimulus	Response to stimulus
Signal transduction	Signal transduction
Single organism cellular process	Single-organism cellular process
Biological regulation	Regulation of cellular process
Cell adhesion	Single-multicellular organism process
Cell developmental process	
Cellular component organization	
Cellular metabolic process	
Cellular process	
Circulatory system process	
Immune system process	
Macromolecule localization	
Macromolecule metabolic process	
Metabolic process	
Organelle organization	
Protein metabolic process	
Regulation of biological process	
Regulation of cell communication	
Regulation of transport	
RNA metabolic process	

DEGs, differentially expressed genes; ISA, incomplete Seppic adjuvant; QCDC, Quil A, cholesterol, dimethyl dioctadecyl ammonium bromide, and Carbopol.

Table 3. Significant biological processes for DEGs in the treatment of adjuvants which were categorized to the term of "Response to stimulus"

Adjuvants group	Biological process	p-value	No. of genes
ISA adjuvants	Cellular response to stress	2.69E-04	53
	Response to oxidative stress	2.06E-03	15
	Positive regulation of locomotion	4.61E-03	14
	Positive regulation of chemotaxis	7.27E-03	8
	Regulation of chemotaxis	7.27E-03	8
	Chemotaxis	9.95E-03	13
	Taxis	9.95E-03	13
	Positive regulation of behavior	1.20E-02	8
	Regulation of behavior	1.20E-02	8
	Response to tumor necrosis factor	1.74E-02	5
	Cellular response to oxidative stress	2.02E-02	6
	Positive regulation of cell migration	3.17E-02	11
	Response to inorganic substance	3.34E-02	9
	Positive regulation of cell motion	3.41E-02	12
	Fibroblast growth factor receptor signaling pathway	3.42E-02	5
QCDC adjuvants	Immune response	9.48E-04	13
	Response to toxin	1.78E-02	3
	Defense response	2.01E-02	8
	Response to bacterium	4.49E-02	5
	Innate immune response	4.85E-02	4

DEGs, differentially expressed genes; ISA, incomplete Seppic adjuvant; QCDC, Quil A, cholesterol, dimethyl dioctadecyl ammonium bromide, and Carbopol.

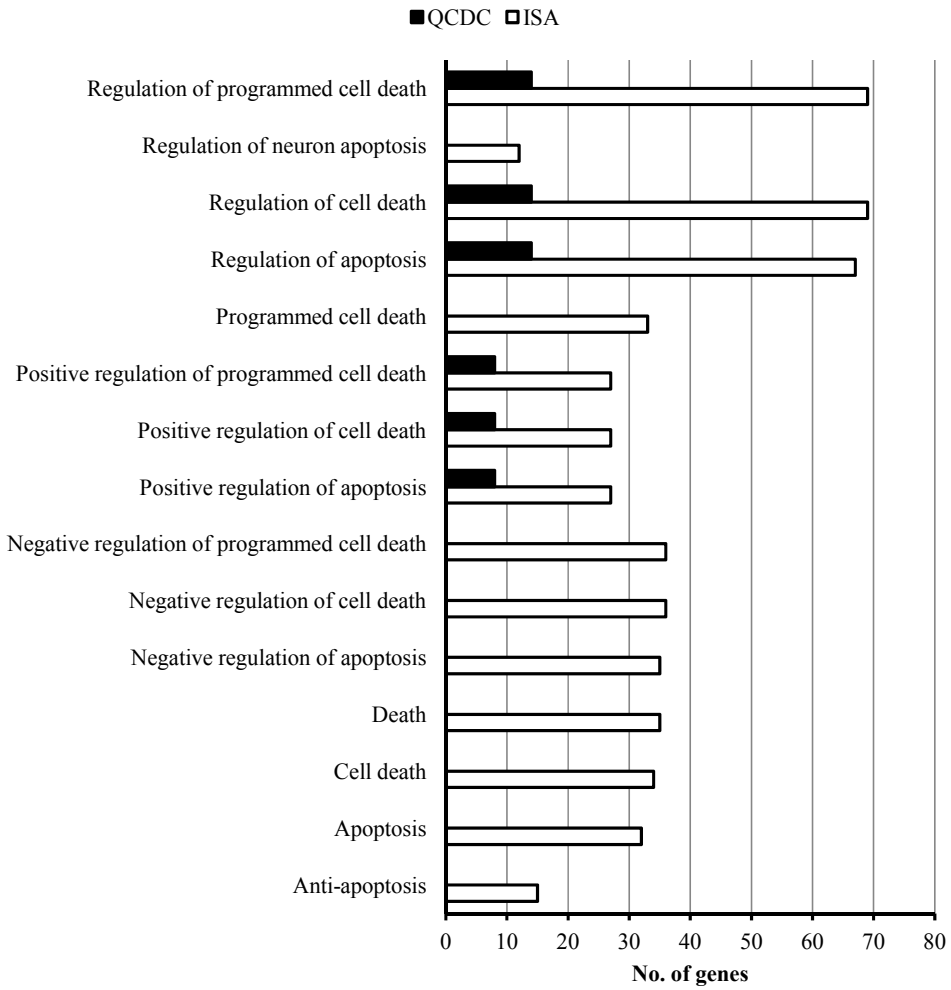


Figure 2. Significant biological processes for differentially expressed genes in the treatment of adjuvants which were categorized to the term of "Cell death"

Pathway analysis

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway terms enriched by DEGs from the treatment of two different adjuvant groups are listed in Table 4. Both adjuvant groups showed common effects on the pathway for "Regulation of actin cytoskeleton". QCDC adjuvants altered genes involved in "Cell adhesion molecules (CAMs)" pathway and ISA adjuvants modified the pathways related with "Spliceosome", "Ubiquitin mediated proteolysis", "Adherens junction", "Lysosome", "Natural

Table 4. Significantly changed pathways from the DEGs in the treatment of ISA adjuvants or QCDC adjuvants

Adjuvants group	KEGG pathway	p-value	Number of genes
ISA adjuvants	Spliceosome	4.34E-04	38
	Ubiquitin mediated proteolysis	5.86E-03	38
	Adherens junction	9.53E-03	24
	Lysosome	1.86E-02	30
	Natural killer cell mediated cytotoxicity	1.93E-02	23
	Cell cycle	2.49E-02	33
	Endocytosis	2.74E-02	47
	Focal adhesion	2.81E-02	49
	Regulation of actin cytoskeleton	3.44E-02	48
QCDC adjuvants	Cell adhesion molecules (CAMs)	3.61E-02	8
	Regulation of actin cytoskeleton	4.91E-02	11

DEGs, differentially expressed genes; ISA, incomplete Seppic adjuvant; QCDC, Quil A, cholesterol, dimethyl dioctadecyl ammonium bromide, and Carbopol; KEGG, Kyoto Encyclopedia of Genes and Genomes.

killer (NK) cell mediated cytotoxicity", "Cell cycle", "Endocytosis", and "Focal adhesion".

DISCUSSION

For the comparative analysis of the effects of two different adjuvant types, we collected microarray datasets from the independent trials for each adjuvant groups, ISAs and QCDCs, and identified GO terms and pathways modified by the DEGs.

In the present study, the most enriched categories of BP affected by two adjuvants, showing 6 categories that are commonly affected by two adjuvants. But, further detailed analysis revealed that the categories consisting of the same BPs in both adjuvants are quite specific, rather than common. For example, in the term of "Response to stimulus", no BPs was identified simultaneously in the two different adjuvant groups (Table 3). Generally, the QCDC adjuvants showed effects on innate immune response and immune response to external stimulus, such as "Response to toxin" and "Response to bacterium". For the immune response to toxins, a good humoral immune response is required in the adjuvants. Most adjuvants on the market today mainly activate the humoral immune response. Nevertheless, it is obvious that the introduction of cell-mediated immune response by adjuvants is beneficial to control bacterial infection, where cytotoxic T cells and Th1 cells mediate these responses (Leclerc, 2003). In this sense, ISCOM, a component of QCDC adjuvants, has been known to generate both strong humoral and cellular immune responses in an extensive range of animal species (Drane et al., 2007). The ISCOMATRIX adjuvant has also shown to be safe and well tolerated as well as immunogenic, generating both antibody (Ab) and $CD4^+$ and $CD8^+$ T cell responses (Drane et al., 2007). Also, DDA is reported to promote both strong cell mediated immune responses and humoral immune responses, which are essential for the induction of protective immunity against most diseases (Davidsen et al., 2005).

As for the adjuvants of ISA series, these modified the BPs that regulate cell movement such as cell locomotion, chemotaxis, and cell migration. Although ISA and QCDC adjuvants modified biological functions related to the regulation of cell death and apoptosis, they showed a different manner in mode of action. The effects of ISA adjuvants on cell death were both of positive and negative regulation while the QCDC adjuvants had no function in regulating cell death negatively. Such apoptotic and necrotic effects are reported to be associated with emulsion-type vaccine adjuvants such as the ISA series and QCDC whereby both are composed of various oils and surfactants (Yang et al., 2004). The mechanism behind the surfactants is that their amphiphilic nature adsorbs not only the oil/water interface in the emulsion, but also biological membranes, resulting in an increase in surface pressure that thus lead to apoptosis and necrosis of the cells (Yang et al., 2004). This suggests that the surfactants in ISA adjuvants and Quil A in QCDC adjuvants could induce apoptosis or cell death during immunization. In the results of pathway analysis, "Adherens junctions" and "Focal adhesion" pathways were the two main types of junction that were modified by ISA adjuvants, which are known to mediate adhesion in epithelial cells (Yeatman, 2004). Cell-cell adherens junctions are important for maintaining tissue architecture and cell polarity and can limit cell movement and proliferation. Cell-matrix adhesions, also called focal adhesions, play essential roles in important BPs including cell motility, cell proliferation, cell differentiation, regulation of gene expression and cell survival. Collectively, the ISA adjuvants may have effects on cell mobility and mitigate cytotoxicity through the cellular junction pathways.

In addition, lysosomal and endocytic reactions were also modified by ISA adjuvants. In the case of lysosomes, the lysosomal enzymes' important role in the inflammatory process has been documented in previous studies in arthritic condition induced by injection of Freund's complete adjuvant (FCA) in rats (Anderson, 1970; Reddy and Dhar, 1988; Geetha and Varalakshmi, 1999). When endocytosis takes place, adjuvants first exert their targeting mechanism by binding to antigens and the adjuvant–antigen complex is delivered to antigen-presenting cells (APCs) to form aggregates that are then engulfed by APCs to form endosomes (Cox and Coulter, 1997; Zamze et al., 2002). More effective targeting can be achieved by using adjuvants with residues that are recognized by receptors on APCs such as the mannose receptor that belongs to the endocytic pattern recognition receptors (PRRs) that bind compounds containing mannose, N-acetylglucosamine, or fucose residues and sulfated oligosaccharides (Stahl and Ezekowitz, 1998).

The ISA adjuvants' functions related with cellular response to oxidative stress shown from our result can be used to partly explain the immune-stimulatory properties of adjuvants that induce inflammation and oxidative stress in the host animal (Kumar and Roy, 2007). An example can be drawn from previous studies that showed that intra-articular injection of a well-known adjuvant, i.e., FCA, induced inflammation as well as immune response and produced features that resembled rheumatoid arthritis in humans. Such acute inflammatory response induced by FCA is associated with leukocyte infiltration, mast cell activation, and release of cytokines and free radicals (Nigrovic and Lee, 2005; Yamada et al., 2006). Despite the shared similarity both ISA adjuvants and FCA and FIA (Freund's incomplete

adjuvant) have with respect to antibody responses, the fact that less inflammatory response is induced by ISA adjuvants relative to FCA and FIA needs to be taken into account (Johnston et al., 1991; Leenaars et al., 1994; Leenaars et al., 1995; Leenaars et al., 1998).

In the pathway analysis, QCDC adjuvants altered genes involved in "CAMs" pathway. Inflammatory and immune responses involve adhesive interactions that mediate migration of cells to sites of inflammation and the effector functions of cell within the lesions. Therefore, CAMs, as complex proteins expressed on the cell surface and precisely regulated by cytokines and other biologic response modifiers, are versatile mediators of the complex dynamics of cell interactions in the inflammatory/immune response (Crawford and Watanabe, 1994).

In conclusion, the integrative analysis of microarray datasets from the treatment of two different types of adjuvants, ISA series and QCDC formulations, has provided discriminative and common molecular signatures as well as aided the distinction of immunological functions of the two adjuvant groups in chicken. Although much progress has been undertaken in the formulation of novel adjuvants that augment the immunogenicity of protein vaccines, very little information is available in poultry (Baldridge and Ward, 1997; Gupta and Siber, 1995; Richards et al., 1996). Therefore, this study could contribute to the selection of appropriate adjuvants according to the types of vaccines or diseases as well as the development of efficient vaccine adjuvants in poultry industry. Furthermore, the potent immune adjuvants might promise another usefulness of vaccine as therapeutic agents rather than prophylactic agents.

Limitations such as small sample size and limited case numbers are weaknesses of this study; however, to our knowledge, this is the first integrative approach to identify molecular signatures impacted by adjuvant in livestock, especially in chicken. Therefore, this study could serve as an informative framework to integrated microarray data to identify molecular signatures that can be used to develop precise adjuvants based on their immunological functions.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

ACKNOWLEDGMENTS

The authors thank Dr. Song, Ki Duk and Ahn, Hyun Ju for their significant contribution to this research. This work was supported by a grant from the Next-Generation BioGreen 21 Program (No. PJ01104401), Rural Development Administration, Republic of Korea.

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