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Predicted philogeny, secondary conformational structure, and epitope antigenicity of immunological sequences in poultry

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ABSTRACT. Poultry production is faced with different types of stresses that are responsible for issues of animal welfare as well as for economic losses. Moreover, the immunity decreases when animals are stressed. *In silico* analyses are important in reducing the cost and in increasing the accuracy of scientific results. A bioinformatics tool was used to perform ontology studies on 15 different immunological sequences of poultry. The mRNA structures and sequences with maximum antigenic residues were also predicted. No homology was found between the sequences of poultry and mammals. These results helped in the prediction of new potential molecular markers. Of the 15 sequences that were analyzed, predictions could not be made for five because they were longer than 2500 nucleotides; for the remaining 10 sequences, 20 conformational

structures per sequence were predicted and the most stable sequences were identified by their minimum free energy values. The highest antigenic epitopes were accepted by the maximum scores; 15 of the total 8934 epitopes that were predicted were analyzed. These results would aid future studies that use synthetic peptides or recombinants as markers or immunomodulators and would expand our understanding on how stress can modulate the immune system. These would also help in developing rapid diagnostic tools, in increasing animal welfare, biosecurity, and productivity, and also in developing of food additives and environmental enrichment for stress control, thereby, making animal production more sustainable.

Key words: Poultry; Bioinformatics; mRNA; Antigenicity; Epitope; Ontology

INTRODUCTION

Stress is a common problem in animals and humans. Negative impacts of stress on poultry industry, such as on their production and quality, and the resulting economic losses, have been described for many years (Mashaly et al., 2004; Rozenboim et al., 2007; Quinteiro-Filho et al., 2010; de Haas et al., 2013; Lara and Rostagno, 2013). Developed countries, such as the United States, incur costs of 1.69 to 2.36 billion dollars on livestock industry per year because of stress (St-Pierre et al., 2003). The release of catecholamines (norepinephrine and epinephrine) (Lyte et al., 2011; Asano et al., 2012) and glucocorticoids (Sapolsky et al., 2000; Hart and Kamm, 2002) in response to stress involves the hypothalamic-pituitary-adrenal (HPA) axis (Gu et al., 2012). Moreover, studies have shown relationships between the different systems, such as neuroendocrine, immune, and gastrointestinal systems (Ogłodek et al., 2014; Robert and Labat-Robert, 2015) in the response against stress.

Immune response can be divided in two different types, namely the innate and adaptive immune response. Generally, the immune system is able to identify and generate a response against anything that causes an imbalance of the homeostasis, such as pathogens, allergens, or any antigen. Innate immune response is the first to recognize the generic patterns of antigens and it continues until the homeostasis is restored. When the innate response is not able to tackle the imbalance caused by the antigen, the adaptive immune response is initiated and the immunity generated by it confers a memory for this particular antigen (Korver, 2012). It is difficult to understand how the receptors and components of the immune system interact to modulate the immune response against stress. This understanding is crucial for identifying the possibilities for modulating the immunity.

During the last decade considerable progress has been made in the field of molecular biology, especially in the studies on RNA that are crucial to understand as to how the immune components modulate the cells and their mechanisms (Frellsen et al., 2009). The prediction and analysis of the conformational structures of molecules provides new perspectives for the understanding of a molecular pathway. These studies can provide clues about the modulation of stress-mediated imbalance by molecules, such as neurotransmitters and immune peptides. For this purpose, molecular tools are important in predicting the structures of molecules. A number of genetic sequences are deposited each year in the public and private databases from a variety

of studies. Bioinformatics is an important tool that can help in the organization and analysis of some of the characteristics of these genetic sequences (ontology) and in the prediction of the secondary conformational structure of mRNAs (Kaikabo and Kalshingi, 2008; Römer et al., 2016). The objective of this study was to evaluate the main immune components and predict their ontology and secondary conformational structure. The aim of this study was to find possible immune molecular markers and to preview the binding sites for manipulating, in the near future, synthetic or recombinant peptides that are able to modulate the immune system and to decipher their interactions with other systems so as to regulate the acute and chronic stress suffered by poultry. Furthermore, the knowledge obtained from the present study may be used to determine the requirement of intervention measures against stress and the best time to implement them, as well as in defining the strategic points of management and biosecurity, in addition to the potential therapeutic targets for prevention and treatment of stress.

MATERIAL AND METHODS

A literature search was performed for identifying the potential molecular markers that could provide a link between the stress response and the immune components (Table 1) using the mRNA from *Gallus gallus*. The markers derived from the innate and acquired immune response were selected for analysis using computational approaches. To measure the inheritance of the genetic sequences within the species, ontology analyses were done. For all the selected genes, a BLAST search was performed and a phylogenetic tree was constructed. Moreover, for all the selected genes, a prediction of the secondary mRNA was made using an online software (http://rna.urmc.rochester.edu/RNAstructure Web/Servers/Fold/Fold.html) following the RNA Fold algorithm to predict the lowest free energy conformation (Mathews et al., 2004). Thereafter, we predicted the epitope antigenicity of all the sequences that were searched. Briefly, the nucleotide sequences were translated into protein sequences and then the antigenicity of the epitopes was determined using an Immune Epitope Database Analysis (http://tools.immuneepitope.org/tools/bcell/iedb_input) following the method of Kolaskar and Tongaonkar (1990).

Table 1. Selected mRNA sequences.

GenBank accession No.	Immune component	Immune response	Query length
gi.208964709	HSP70	I	1920
gi.45433513	IL1	I	1107
gi.971376440	IL1-R	I	2017
gi.55741695	IL4	A	411
gi.971419656	IL4-R	A	3764
gi.148540095	IL10	I	528
gi.88853848	IL10-R	I	2189
gi.47087194	IL12	I	948
gi.478476198	IL12-R	I	2678
gi.45433517	INF γ	I	1330
gi.62899051	INF γ-R	I	2221
gi.2707470	TCR γδ	I, A	306
gi.71897340	TLR4	I	3532
gi.53854909	TNF α	I	674
gi.66793458	TLR5	I	2586

Table 1. Selected mRNA sequences. I (Innate), A (Adaptive), HSP (Heat shock protein), IL (Interleukin), R (Receptor), INF γ (Interferon gamma), TNF α (Tumor necrosis factor alpha), TCR γδ (T cell receptor gamma delta), TLR (Toll like receptor).

RESULTS AND DISCUSSION

After the analysis of all the gene sequences using the bioinformatics tools described above, we identified that some genes, such as those of cytokines did not form any phylogenic cluster (**Figures S1-S15**). The majority of cytokines receptors and toll like receptor (TLR) 4 and 5 had just one root in common, specifically for TLR 4 and 5 because they recognize the same patterns (Leveque et al., 2003; Kogut et al., 2006). Only interleukin (IL) 1 had three genetic clusters and T cell receptor gamma had six genetic clusters. This can be explained based on the fact that mammals and poultry had different ancestors and they evolved differently. Instead, gene homology can be found for humans and a lack of mammals contributing to the use of same cell molecular marker in different species (Schulpen et al., 2015). The rodents, such as mice and rat, are important models for several human diseases because they have high gene homology and the results obtained in the rodent models can be extrapolated for humans (Huang et al., 2016). It explains the importance of this study where the molecular markers in poultry are so different from that in a common ancestor and demonstrates no homology between the genes.

Depending on the evolutionary distance between the phylogenic clusters, molecular markers described in the literature can be used because domains in the members of a cluster are conserved. However, when the markers do not belong to the same phylogenic cluster the target sequence needs to be checked for possible homology between the studied species in order to increase the accuracy of the molecular marker (Igea et al., 2010). From our perspective, this ontology study was crucial for determining whether we could use the existing molecular markers for the mammalian immune system or we would need to search for potential immune molecular markers specific for poultry. Our results show the importance of selecting the potential immune genes, and of analyzing and testing them for complete understanding of how and which immune response of poultry can be modulated by stress. The interleukin receptor can have the same root as those of the other species because they are more conserved than the soluble interleukins.

Minimum free energy (MFE) analysis (Table 2) revealed a low energy for each mRNA sequence. This suggests that at a controlled temperature T (310.1 K = 37ºC) the more stable conformational molecule structure will have the best predicted protein following the central dogma of molecular biology (RNA-Protein) (Jaeger et al., 1989). Among the 15 mRNA sequences, MFE could only be predicted for 10; the remaining five sequences had more than 2,500 nucleotides and could not be analyzed by the software. We determined 200 values of MFE (20 per sequence) and the lowest MFE values were selected (**Figures S16-S25**) so as to ensure that mRNAs with the most stable secondary structures are studied in the future (Drory Retwitzer et al., 2015; Fu et al., 2015).

The results of our ontology analysis revealed that the sequences identified by us did not have any regions that were conserved in other molecular markers, which have already been studied, highlighting the fact that this is a pioneering study on the structures of nucleotides of genes involved in immunity in poultry. Other studies have shown the prediction of mRNA structure for comprehending the tertiary mRNA structure and the positive or negative impacts of the resulting protein on the organism (Marashi et al., 2006; Frellsen et al., 2009; Bida and Maher, 2012; Contrant et al., 2014) as well as its use in manipulating genetic sequences, for example, to create new recombinant vaccines (Ilyinskii et al., 2009).

Table 2. Prediction of minimum free energy of mRNA.

Immune component	Immune response	MFE
HSP70	I	-6337
IL1	I	-431
IL1-R	I	-5332
IL4	A	-1261
IL10	I	-1757
IL10-R	I	-8723
IL12	I	-248
INF γ	I	-2976
INF γ-R	I	-6652
TNF α	I	-2078

MFE (Minimum free energy), I (Innate), A (Adaptive), HSP (Heat shock protein), IL (Interleukin), R (receptor), INF γ (Interferon gamma), TNF α (Tumor necrosis factor alpha).

More recently, studies on epitope antigenicity have been performed with the aim of reducing the costs and achieving higher accuracy of new diagnostic platforms. Our antigenicity results (Table 3) and the results shown graphically (**Figures S25-S40**) revealed a total of 8,934 different antigenic epitopes. The maximum antigenicity score was taken for each sequence/protein. We wanted to get the maximum score for each epitope so as to get the most stable structure of mRNA that would lead to the most antigenic epitope for building our immunodiagnostic platform for assessing stress in poultry. Other studies have shown a high performance using this analysis and the determination of the best antigenic epitope relevant for an immunodiagnostic platform, vaccine production, and several treatments, especially in humans (Kolaskar and Tongaonkar, 1990; Hernández-Guzmán et al., 2014; Cai et al., 2015; Gomez et al., 2015). Such results could start the process for understanding of the immunomodulation caused by stress.

Table 3. Prediction of epitope antigenicity.

Immune component	Immune response	Number of epitopes	Position	Residue	Peptide	Score
HSP70	I	628	443	V	SVLVQVY	1.227
IL1	I	195	100	F	SAFFLFC	1.144
IL1-R	I	1030	16	I	AVIICVV	1.276
IL4	A	771	712	P	VVVPRAV	1.219
IL4-R	A	298	270	P	ALGPVVL	1.181
IL10	I	169	7	A	CCQALLL	1.236
IL10-R	I	561	237	L	VFVLLIL	1.251
IL12	I	356	11	V	ACCVVLA	1.281
IL12-R	I	1861	620	P	LYIPCVV	1.258
INF γ	I	158	11	L	LFVLSVI	1.217
INF γ-R	I	561	237	L	VFVLLIL	1.251
TCR γδ	I, A	642	383	Y	VICYILV	1.270
TLR4	I	787	614	L	ILILVVV	1.279
TNF α	I	142	121	I	CCLIPFC	1.256
TLR5	I	775	602	L	ILILVVV	1.279

I (Innate), A (Adaptive), HSP (Heat shock protein), IL (Interleukin), R (Receptor), INF γ (Interferon gamma), TCR γδ (T cell receptor gamma delta), TLR (Toll like receptor), TNF α (Tumor necrosis factor alpha).

CONCLUSIONS

This study provided 21 mRNA sequences with the highest epitope antigenicity, minimum free energy and specific for poultry which may have a high potential to be an immune molecular marker. Our work limitation is only predicted results, but it is the first

project on this line and these in silico analysis had low cost compared with in vitro or in vivo experiments. At this point we can go further on this project which may lead an elucidating of stress diagnostic and better understand stress misbalance.

Conflicts of interest

The authors declare no conflicts of interest.

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Supplementary material

Figure S1. Phylogenetic tree of *Gallus gallus* Heat shock protein.

Figure S2. Phylogenetic tree of *Gallus gallus* Interleukin 1 beta.

Figure S3. Phylogenetic tree of *Gallus gallus* Interleukin 1 beta receptor.

Figure S4. Phylogenetic tree of *Gallus gallus* Interleukin 4.

Figure S5. Phylogenetic tree of *Gallus gallus* Interleukin 4 receptor.

Figure S6. Phylogenetic tree of *Gallus gallus* Interleukin 10.

Figure S7. Phylogenetic tree of *Gallus gallus* Interleukin 10 receptor.

Figure S8. Phylogenetic tree of *Gallus gallus* Interleukin 12 receptor.

Figure S9. Phylogenetic tree of *Gallus gallus* Interleukin 12.

Figure S10. Phylogenetic tree of *Gallus gallus* Interferon gamma.

Figure S11. Phylogenetic tree of *Gallus gallus* Interferon gamma receptor.

Figure S12. Phylogenetic tree of *Gallus gallus* T cell receptor gamma delta.

Figure S13. Phylogenetic tree of *Gallus gallus* Toll like receptor 4.

Figure S14. Phylogenetic tree of *Gallus gallus* Toll like receptor 5.

Figure S15. Phylogenetic tree of *Gallus gallus* Tumor necrosis factor-alpha.

Figure S16. Minimum free energy of *Gallus gallus* Heat shock protein mRNA.

Figure S17. Minimum free energy of *Gallus gallus* Interleukin 10 mRNA.

Figure S18. Minimum free energy of *Gallus gallus* Interleukin 1 receptor mRNA.

Figure S19. Minimum free energy of *Gallus gallus* Interleukin 1 mRNA.

Figure S20. Minimum free energy of *Gallus gallus* Interleukin 4 mRNA.

Figure S21. Minimum free energy of *Gallus gallus* Interleukin 10 receptor mRNA.

Figure S22. Minimum free energy of *Gallus gallus* Interleukin 12 mRNA.

Figure S23. Minimum free energy of *Gallus gallus* Interferon gamma mRNA.

Figure S24. Minimum free energy of *Gallus gallus* Interferon gamma receptor mRNA.

Figure S25. Minimum free energy of *Gallus gallus* Tumor necrosis factor-alpha mRNA.

Figure S26. Epitope antigenicity of *Gallus gallus* Heat shock protein.

Figure S27. Epitope antigenicity of *Gallus gallus* Interleukin 1 receptor.

Figure S28. Epitope antigenicity of *Gallus gallus* Interleukin 1.

Figure S29. Epitope antigenicity of *Gallus gallus* Interleukin 4 receptor.

Figure S30. Epitope antigenicity of *Gallus gallus* Interleukin 4.

Figure S31. Epitope antigenicity of *Gallus gallus* Interleukin 10 receptor.

Figure S32. Epitope antigenicity of *Gallus gallus* Interleukin 10.

Figure S33. Epitope antigenicity of *Gallus gallus* Interleukin 12 receptor.

Figure S34. Epitope antigenicity of *Gallus gallus* Interleukin 12.

Figure S35. Epitope antigenicity of *Gallus gallus* Interferon gamma receptor.

Figure S36. Epitope antigenicity of *Gallus gallus* Interferon gamma.

Figure S37. Epitope antigenicity of *Gallus gallus* T cell receptor gamma delta.

Figure S38. Epitope antigenicity of *Gallus gallus* Toll like receptor 5.

Figure S39. Epitope antigenicity of *Gallus gallus* Tumor necrosis factor-alpha.

Figure S40. Epitope antigenicity of *Gallus gallus* Toll like receptor 4.