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Association Analysis Between Polymorphisms of *Nramp1* Gene and Immune Traits in Chicken

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Abstract: The present research was to study the association of polymorphism of Natural resistance-associated macrophage protein1 (Nramp1) with some immune functions in Rugao chicken (RG) and Recessive White chicken (RW). The PCR-SSCP technique was applied to analyze the correlation between the polymorphisms of Nramp1 gene and immune functions (Heterophil/Lymphocyte (H/L), lymphocyte transformation rate and the content of IgM) in 72 RG and 55 RW. The results showed that: the Heterophil/Lymphocyte (H/L), lymphocyte transformation rate and the content of IgM in RG and RW showed significant differences ($p<0.05$). H/L of AA was significantly lower than BB and AB while the AA's lymphocyte transformation rate and IgM were significantly higher than the BB's in both RG and RW. The results demonstrated that the general immune performances of RG were superior to those of RW. The general immune performances of AA were superior to BB and AB.

Key words: Chicken, SSCP, *Nramp1* gene, immune function, polymorphisms, China

INTRODUCTION

Disease was an important factor in restricting the development of animal husbandry. With the development of Animal Husbandry, various mixed diseases and new diseases continued to take place and circulate (Liao, 1996). It was often found that resistance to disease was significantly different between varieties in practices. This difference in disease resistance also constituted the genetic basis of selection (Shi, 2004). Resistance traits were affected by many factors, resulted in the genetic progress of conventional breeding methods is very slow so many researchers have done a lot of research centering on the work of the candidate genes in order to look forward to using genetics to improve disease resistance of animal. *Nramp1* gene which is a major candidate gene of disease resistance has been widespread concerns (Liu *et al*., 2003). Rugao chicken and Recessive chicken which belonged to Chinese native chicken breeds and introduced breeds, respectively were selected to study in this experiment.

In this study, it was detected some immune functions such as the heterophilic cell and lymphocyte cell ratio (H/L), lymphocyte transformation rate, IgG and IgM levels. Meanwhile, SNPs in exon 9 of the *Nramp1* gene was detected by SSCP and got each unique genotype by using DNA sequence analyses. Finally, it was analyzed the association between each genotype and its immune functions among two breeds (RG and RC) and aim to provide the theoretical basis for developing new chicken resistant lines.

MATERIALS AND METHODS

Chicken and DNA sources: There were 127 samples from two breeds (Rugao chicken n = 72; Recessive chicken n = 55) investigated in this study. These samples were collected from Institute of Poultry Science. Genomic DNA utilized in PCR amplification was isolated from frozen blood or tissue samples by using a conventional phenol-chloroform extraction procedure as described by Blin and Stafford.

PCR amplification: According to chicken *Nramp1* gene (GenBank accession number: AY072007), a pair of primers were designed to amplify exon 9 of chicken *Nramp1* gene. The sequences of the primers are as follows:

- Forward: 5'CCATGGTGTACATTACCTGCC'3
- Reverse: 5' TCCACCCACCTAACCAGACTA'3

The 25 μL volume contained 50 ng genomic DNA, 0.6 μM of each primer, 1.5 mM $MgCl_2$, 200 μM dNTPs and 0.5 units of Taq DNA polymerase. The cycling protocol

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was 5 min at 95°C, 35 cycles of denaturing at 94°C for 30 sec, annealing at 55°C for 30 sec, extending at 72°C for 40 sec with final extension at 72°C for 10 min.

SNPs analyses: A 3 μL aliquot of each amplimer was mixed with 7 μL denaturing solution (95% formamide, 10 mM EDTA, 0.025% xylene-cyanol, 0.025% bromophenolblue), heated for 10 min at 98°C and cooled rapidly on ice and then loaded on 16×18 cm 10% acrylamide: bisacrylamide (29:1) gels. Electrophoresis was performed at 120 V for 12 h in 1× TBE buffer. The gel was stained with 0.1% silver nitrate (An *et al*., 2009). Amplimers representative of each unique SSCP banding pattern (homozygote) were purified, cloned, screened and then were sent to the sequencing company (Sangon Biotech, Shanghai) to sequence.

The principle and method of the immunity index detection: The value of H/L was detected followed Campo's method (Campo and Davila, 2002). MTT (methyl thiazolyl tetrazolium) chromatometry was used to detect lymphocyte transformation (Li *et al*., 2006; Wang *et al*., 2006). ELISA was used to detect the value of IgM.

Statistical analytic method: SPSS (Version 16.0) was used to analysis the date. The following linear model was used to analyze the association between the immune functions and genotypes:

$$y_{ijk} = \mu+B_i+S_j+G_k+B_i\times S_j+B_i\times G_k+S_j\times G_k+B_i\times S_j\times G_k+e_{ijk}$$

where, y, B, S, G, B×S, B×G, S×G, B×S×G and e are vectors of observations, breed effects, sex effects, genotype effects, breed and sex reciprocal effects, breed and genotype reciprocal effects, sex and genotype reciprocal effects, breed, sex and genotype reciprocal effects and random environmental residual effects, respectively.

RESULTS AND DISCUSSION

Immune functions of different varieties: Table 1 showed that the values of H and H/L of rugao chicken were less than the recessive chicken while the value of L was higherthan the recessive chicken. Lymphocyte transformationrate and the value of IgM of rugao chicken were higher than the recessive chicken. From the mean point of view, lymphocyte transformation rate and the value of IgM were 12.66% and 35.8 pg mL^{-1} higher than recessive chicken.

Immune functions of different genotypes: All the chicken genomes of the experimental groups were amplified and the PCR products were detected with 1.5% agarose gel. The product obtained was the same as the target fragment and the product strap was clear without mixed straps. Thus, the product was amplified with fine stability and specificity and can be cut by restriction enzymes directly (Fig. 1). This fragment having 3 genotypes which were named AA, AB and BB by detection of SSCP (Fig. 2). The fragment of 2 chicken which were representatives from the three determined *Nramp1* genotypes was sequenced and 2 SNPs which were C315G and C357T were then found. Genotype AB is the predominant type in the set of analyzed chicken (47.2% in RG and 54.5% in RW Table 1). Frequency of allele A is high than that of B (Table 2). The means of five important immune functions (H, L, H/L, lymphocyte transformation and IgM) were calculated with SPSS 16.0 by the founded model (Table 3). The value of H and the value of H/L of RW were less than the other 2 genotypes while the value of L, lymphocyte transformation and IgM were greater than the other two genotypes. All of the indicators of AB genotype were in the middle of the other two genotypes. Various kinds of values of the indicators in RG were the same trend like what were in RW. Researchers found the immune traits between different genotypes was significant, herein, the mean of AA was greater than that of the other two genotype ($p<0.05$).

Lymphocyte, one of leukocyte which is produced by the lymphoid organ, plays an important role in immune responses. The measurement of H/L value was not only commonly used in the clinical diagnosis of veterinarian but also in the resistance breeding of poultry as a main immune parameter with high heritability (Al-Murrani *et al*., 1997). It was previously reported that H/L value was increased by mild or moderate stress (Maxwell, 1993) and the ratio of H/L for Beijing fatty chicken was less than

Table 1: Immune performances of different breeds

Breeds	Number	Heterophil (H)	Lymphocyte (L)	Heterophil/ Lymphocyte (H/L)	Lymphocyte transformation	I gM (pg mL^{-1})
RW	55	27.05±1.58^a	72.95±1.58^a	0.38±0.014^a	21.57±1.16^a	100.95±6.33^a
RG	72	17.04±0.99^b	82.96±0.99^b	0.22±0.016^b	34.23±2.23^b	136.75±4.41^b

Values with different superscripts within the same column differ significantly at $p<0.05$

Table 2: Genotypes and allele frequencies of *Nramp1* gene

	RG				RW			
Genotype	No.	Frequency	Allele	Frequency	No.	Frequency	Allele	Frequency
AA	29	0.403	A	0.639	15	0.273	A	0.546
AB	34	0.472	B	0.361	30	0.545	B	0.454
BB	9	0.125	-	-	10	0.182	-	-

Table 3:Correlation analysis between *Nramp1* genotypes and immune performances

Breeds	Genotype	Heterophil (H)	Lymphocyte (L)	Heterophil/ Lymphocyte (H/L)	Lymphocyte transformation (%)	IgM(pgmL^{-1})
RW	AA	18.25±1.24^{a}	81.75±1.24^{a}	0.20±0.016^{a}	27.36±1.73^{a}	136.25±12.45^{a}
	AB	28.67±2.40^{b}	71.33±2.40^{b}	0.43±0.070^{b}	22.04±1.66^{b}	97.04±8.020^{b}
	BB	40.43±2.30^{c}	59.57±2.30^{c}	0.63±0.070^{c}	12.69±1.29^{c}	82.65±12.85^{b}
RG	AA	12.85±0.79^{a}	87.15±0.79^{a}	0.15±0.011^{a}	42.17±2.79^{a}	151.27±5.530^{a}
	AB	18.94±1.45^{b}	81.06±1.45^{b}	0.25±0.023^{b}	30.41±3.74^{b}	121.34±8.680^{b}
	BB	26.67±3.67^{c}	73.33±3.67^{c}	0.39±0.067^{c}	26.34±5.58^{b}	134.72±6.960^{b}

Values with different superscripts within the same column differ significantly at $p<0.05$

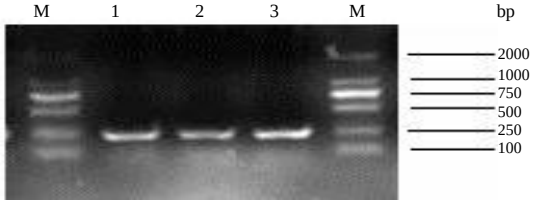

Fig. 1: PCR products of *Nramp1* gene. M: DL 2000 bp DNA marker

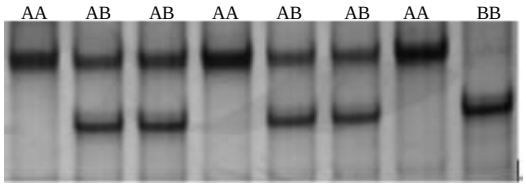

Fig. 2: Polyacrylamide gel electrophoresis of three genotypes: 1, 4, 7: AA; 8: BB; 2, 3, 4, 5, 6: AB

white leghorn chicken's, suggesting the resistance of the latter surpasses the former (Wu and Zhao, 2007). In this research, it was obviously less in RG than that in RW, indicating that under the same condition, RG in the abilities of anti-stress and anti-resistance as well as the specific and non-specific immune functions with resisting pathogenic microorganism were stronger than RW. Furthermore, it proved thatanother index, lymphocyte transformation rate which elevation may strengthen organism immunity (Li and Zhao, 2003). Because co-culture of lymphocyte with many non-specific antigens *in vitro* transforms into lymphoblast and according to the level of proliferation and differentiation of lymphocyte can speculate the immunologic function. This experiment demonstrated that the rate in RG exceeded RW evidently which may further account for indigenous breed was superior to introduced breed on resistance. The immunoglobulin is functionally or structurally similar to the antibody and compared with others, IgM mainly shows immunologic function at early stage of infection. As was seen in mouse, the level of IgM in the ones with immunodeficiency after being infected was significantly lower than other control groups (Sun, 2007). Here, based on database analyses it revealed that this value in RG was far more than RW.

Taking all of these parameters mentioned above into consideration, it is conceivable that RG has more advantages than RW in disease-resistant and anti-stress abilities which confirms the different immunologic function among the varieties and provides the scientific evidence for the cultivation of new disease-resistant breeds. Since the mechanism of mutated *Nramp1* gene resulting in disruption of arresting salmonella typhimurium in mouse has been known to the public, the scholars for molecular breeding wonder whether it is in common with other organisms. In pig, the difference for cytotoxicity of monocytes between different genotypes was remarkable with the BB genotype overtakes AB genotype ($p<0.05$) (Wu *et al*., 2008).

A significant association between distinct genotype and the immunologic function was found in Chinese Holstein thus, the *Nramp1* gene might be taken as critical candidate gene for the disease-resistant character (Hu *et al*., 2009). However, there were few reports about *Nramp1* gene in chicken. In this research, two mutations, C315G and C357T, respectively were discovered in the 9 exon in both RG and RW for the first time. Analysis of correlation between the polymorphisms of *Nramp1* gene and immune functions demonstrated that H/L value of AA genotype was markedly less than BB genotype and AB genotype while the AA genotype for lymphocyte transformation rate and IgM were notably more than the BB genotype's in both breeds.

CONCLUSION

Overall, the general immune performance of AA genotype was superior to BB genotype and AB genotype. Thus, the AA might be regarded as the genotype with potentially high resistance and contribute to molecular marker-assisted selection on the disease-resistant breeding for cultivation of novel varieties.

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