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Sequence variation in GAL1 and GAL2 genes in Khuzestan local chickens

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Abstract

Beta defensins were small, cationic peptides that bind to microbial membranes and disrupt their integrity, thereby exerting antimicrobial effects. These peptides play an important role in innate immunity against microbial pathogens. The aim of the present study was to detect sequence variations, if any, in some beta-defensin gene (GAL1 and GaL2) sequences in Khuzestan local chickens. Blood samples were collected from 20 local chickens and the genomic DNA was isolated using the standard phenol chloroform method. To detect sequence variation, the interested regions of GAL1 and GAL2 were amplified by specific primers with polymerase chain reaction (PCR). Then, the PCR products were sequenced in both directions. Analysis of the GAL1 gene found many sequence variations in the promoter region. GAL2 gene sequencing determined various sequence variations in intronic and exonic regions. These results suggest the existence of numerous genetic variations in these genomic sequences. Further association studies may help the development of a PCR-based genotyping test to select parents with a better immunity.

Keywords: Beta Defensins Genes, Sequence Variations, Immunity, Khuzestan Local Chickens

Introduction

Most studies in poultry science are carried out for industrial sectors. The village sector is less studied, even though it usually supports indigenous breeds that are maintained for a few years during a specific area and adapt to indigenous conditions. These native chickens can form the basis for genetic improvement and diversification to create breeds modified for local conditions(Sørensen, 2010). Unfortunately, 500 chicken breeds are classified as being in danger. Despite their low growth rates and egg production, indigenous chickensare immune to various diseases and may survive under harsh nutritional and environmental circumstances(Minga et al., 2004). In numerous developing countries in Africa and Asia, native chickens represent up to eightieth of the national flocks(Sørensen, 2010). In Iran, native chickens are of the meat-egg or dual-purpose type. However, compared to industrial breeds, they are typically poor producers of eggs and meat(Gorgani Firozjah et al., 2015). In the Southern part of Iran, particularly in Khuzestan Province where heat stress is one of the most important abiotic stresses, indigenous chickens are grown in all villages. Like other developing countries, the importation of commercial breeds has increased the risk of extinction in Iranian native chickens throughout the past century(Ghazikhani-Shad et al., 2007). Accordingly, lack of attention to indigenous chickens and the increasing use of foreign industrial strains from developing countries raise a serious threat to the future of indigenous chickens and, finally, will result in their total disappearance(Ansari et al., 1997).

According to different reports, native chicken populations can be considering in future animal breeding programs in the world(Makarechian et al., 1983). Therefore, the utilization of

animal breeding programs with the aim of extending the genetic potential of indigenous chickens has been considerable in developing countries(Tadelle et al., 1999). In addition to being valuable gene pools or major supplies of genes, indigenous chickens are able to improve the quality of products through heterosis or morphological and physiological changes(Ansari et al., 1997; Tadelle et al., 1999). In poultry production fields, diseases cause huge problems such as high morbidity and mortality; negative effects on the efficiency of production(Biggs, 1982); high costs of disease prevention, vaccination, and medical treatments; and food-safety problems in human consumption due to bacterial diseases and antibiotic use in animal food(Klasing and Korver, 1997). Since, planning to use genetic selection and breeding program for resistance to disease using molecular markers would result in a resistance population and reduce the price of treatment(Pruthviraj et al., 2016). In breeding programs, when using popular techniques such as those relying upon to polymerase chain reaction (PCR), it is better to consider molecular markers that are close to health-related peptides' ordination. To date, some studies have been conducted to find the connection between poultry genome and poultry diseases (Kramer et al., 2003); however, beta-defensin genes have been neglected.

Beta-defensins belong to a family of defensins which is ataxonomic group of cationic antimicrobial peptides (AMPs) with broad-spectrum antimicrobial activity against various microorganisms, fungi, and viruses(Lai and Gallo, 2009). Defensins are antimicrobial peptides that play a vital role in innate host defense (Higgs et al., 2005; Sugiarto and Yu, 2004). Of the 3 defensins subfamilies (alpha, beta, and theta-defensins), only beta-defensin (gallinacins) has been found in poultry(Sugiarto and Yu, 2004; Xiao et al., 2004). Beta-defensin genes in humans, cows, and poultry have been found to be related to many diseases (Hasenstein and Lamont, 2007). They are comparatively small antimicrobial peptides (typically less than 100 amino acids in size) which possess a broad range of antimicrobial activities (Sugiarto and Yu, 2004). Currently, within the sequence of chickens, thirteen different beta-defensin genes have been designated as gallinacin (Gal)1 to 13 (Xiao et al., 2004) and mapped within an 86-kb region of chromosome 3q3.5-q3.7 (Xiao et al., 2004). In chickens, gallinacins 1 to 13 possess an identical genomic structure of 4 short exons which are separated by 3 introns with different lengths (Xiao et al., 2004). These peptides are abundant in cells that are involved in the innate immunologic response(Xiao et al., 2004) and exhibit different antimicrobial activities against Gram-positive and Gram-negative microorganisms (Higgs et al., 2005).

Given that native species can be a potential source of genetic resources especially in breeding for disease resistance, Khuzestan native chickens should be given special consideration because they are reared cheaply in the free range system and adapt to local environmental conditions, and the cost of treating their diseases is very low. Although this type of chicken is characterized by low productivity, farmers keep it for domestic consumption and sell the excess for extra family income. Nevertheless, it might be a possible reservoir and potential means of disease resistance in poultry breeding for poultry commercial sectors especially in Iran. On the other hand, studies on genes responsible for immunity would aid the selection for disease resistance. Therefore, the objective of this study was to assess genetic variability, if any, in genomic regions of GAL1 and GAL2 genes among Khuzestan native chickens.

Material and methods

Khuzestan climate and experimental animals

The sequence variation study was conducted on domestic chickens belonging to *Gallus gallus* species reared at a local poultry breeder, Dasht-e Azadegan, Khuzestan, Iran. Khuzestan Province, situated in the Southwest of Iran, has an area of 64075 km^2, with more than 4000 villages,

located between 47°41′ to 50°39′ of the eastern longitude from the Prime Meridian and 29°58′ to 33°04′ min of the northern latitude from the Equator. The climate of Khuzestan is mostly very hot and infrequently wet, particularly within the South, while winters are cold and dry. Summertime temperatures routinely exceed 45 °C (113 °F) and fall below 0 °C in the winter. Khuzestan is possibly among the hottest places on Earth with maximum temperature in the summer soaring up to 55 °C (131 °F), rising up to about 60 °C at times.

Collection of blood samples and extraction of DNA

Five mm of blood was gathered from 20 Khuzestan native chickens (after slaughtering) into a sterile 15mm plastic centrifuge tube containing ethylene diamine tetra acetic acid (EDTA) as the anticoagulant medication (1 mg/mL of blood). The tubes were tightly capped and kept in an ice box. Samples were taken to the biotechnology laboratory and held at –20°C until the isolation of DNA. Genomic DNA was isolated from erythrocytes using the standard phenol-chloroform methodology.

PCR amplification

To differentiate sequence variations in GAL1 and GAL2 genes in Khuzestan native chickens, some primers (Table 1) were designed by Oligo7 (Rychlik, 2007) based on the published genomic DNA sequence found in the NCBI database (AY621316.1 andAY621317.1). PCRs were carried out in a thermal cycler (Bio-Rad, Hercules, CA,USA) using 25µL of reaction mixture volumes that contained 1×Taqpolymerase buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP mixture, 10 pM/µL forward and reverse primers, 50 to 100 ng template DNA, 0.75 units Taq DNA polymerase (Solis BioDyne, Tartu, Estonia), and nuclease free water to make up the volume. The following cycling conditions were utilized: an initial denaturation step at 95°C for 5 min, followed by 39 cycles at 95°C for 45 s at the optimal annealing temperature for 30 s (Table 1), and for 1 min at 72°C and a final extension step for 10 min at 72°C.

Table1. Primer sequences used for amplification of fragments of GAL1 and GAL2 genes

Gene (GenBank accession No.)	Primer sequences (forward/reverse)	PCR productsize (bp)	Annealing temp. (°C)	Start-stop
Gal1(AY621316.1)	GCTGTTCTTGGTGGGGTTCT	1195	55	1887-3081
	TCAGAGCCCTCCTAGTCGTT			
Gal2(AY621317.1)	AGCGTGGCAGGAAATCTGAA	1406	55	4687-6073
	GTCTTCTTGCTGCTGAGGCT			

Nucleotide sequencing

In this experiment, the PCR product was created per each blood sample of the chickens. Next, 20 tubes of PCR products were randomly divided into five groups, each containing four PCR products of samples. Finally, 25µL of PCR product from each group was considered for DNA sequencing. Purification and sequencing of PCR products were performed at Macrogen, Seoul, Korea. Sequencing was performed in both directions with 100 nt paired-end reads using the Illumina HiSeq 2000 platform (Macrogen, South Korea). The MEGA5.2 software was used for sequence assembly and identification of the polymorphisms (Tamura et al., 2011).

Results

Since genetic polymorphisms in the upstream region of genes may be in expression regulatory elements, they can enhance or reduce the efficiency of the expression of a relevant structural gene and thus influence the physiological traits of the birds. We studied the 5’ flanking region of the GAL1 gene in Khuzestan native chickens. In the present study, the exonic and intronic

regions of GAL2 gene were considered for sequence variation detection. The exonic region(s) of a gene may contain a mutation(s) that may alter the sequence of amino acids in protein and cause biological and physiological differentiation. On the other hand, although mutations in intronic regions do not influence the formation of protein, these alterations can supply excellent molecular markers for genetic evaluation and animal selection (Singh U. et al., 2014).

Overall, in this study, about 2.4 kb of genomic regions were sequenced in two directions from each of the five groups for detecting sequence variations in GAL1 and GAL2 genes. Sequencing results indicated that a total of 37 point mutations existed in these regions. The mutation rate of these DNA sequences that belongs to Khuzestan native chickens was equal to 15.4 SNPs/kb.

The nucleotide sequence analysis demonstrated that the 5' flanking region of the GAL1 gene in Khuzestan native chickens had mutations (**Ошибка! Источник ссылки не найден.**). In the 1200 bp fragment of the GAL1 gene that had been amplified by PCR, 22 genetic variations occurred. The frequency of mutation in this genomic region was equal to 18.3 SNPs/kb. These mutations were g. -16A>G, g. -26A>C, g. -143T>C, g. -159A>G, g. -187G>T, g. -191T>C, g. -209A>G, g. -420G>C, g. -428T>C, g. -466T>C, g. -488A>C, g. -506T>C, g. -533G>T, g. -654A>G, g. -659G>C, g. -743A>G, g. -772A>T, g. -830T>C, g. -921T>C, g. -924A>G, g. -930T>C and g. -955T>C. Most of these mutations were of the substitution type (A/G=8 and C/T=5).

Table 2. Sequence variation of the upstream region of GAL1 gene in Khuzestan native chickens

Position based on AY621316.1 accession No.	Position based on A of the ATG-translation initiation codon	Mutation base	Genomic region	Mutation name
2928	-16	A>G	Upstream	g. -16A>G
2918	-26	A>C	Upstream	g. -26A>C
2801	-143	T>C	Upstream	g. -143T>C
2785	-159	A>G	Upstream	g. -159A>G
2757	-187	G>T	Upstream	g. -187G>T
2753	-191	T>C	Upstream	g. -191T>C
2735	-209	A>G	Upstream	g. -209A>G
2524	-420	G>C	Upstream	g. -420G>C
2516	-428	T>C	Upstream	g. -428T>C
2478	-466	T>C	Upstream	g. -466T>C
2456	-488	A>C	Upstream	g. -488A>C
2438	-506	T>C	Upstream	g. -506T>C
2411	-533	G>T	Upstream	g. -533G>T
2290	-654	A>G	Upstream	g. -654A>G
2285	-659	G>C	Upstream	g. -659G>C
2201	-743	A>G	Upstream	g. -743A>G
2172	-772	A>T	Upstream	g. -772A>T
2114	-830	T>C	Upstream	g. -830T>C
2023	-921	T>C	Upstream	g. -921T>C
2020	-924	A>G	Upstream	g. -924A>G
2014	-930	T>C	Upstream	g. -930T>C
1989	-955	T>C	Upstream	g. -955T>C

The sequencing results of GAL2 gene in Khuzestan native chickens revealed that this part of the gene contained genetic variations (Table 3). A total of 15 mutations were identified in this region of DNA including g. -85A>C, g. 50T>C, g. 159G>T, g. 164G>T, g. 259C>G, g.263A>G, g. 276A>T, g. 393G>T, g. 399A>G, g. 465A>G, g. 552C>T, g. 601A>G, g. 748C.T, g. 807C>T and g. 1000C>T. The sequence variation rate was equal to 12.5 SNPs/kb. The mutation g. -85A>C occurred in the promoter region of GAL2 gene, while most of these sequence variations occurred in intronic regions (g. 159G>T, g. 164G>T, g. 393G>T, g. 399A>G, g. 465A>G, g. 552C>T, g. 601A>G, g. 748C.T, g. 807C>T and g. 1000C>T) and only four nonsynonymous genetic variations including g. 50T>C, g. 259C>G, g.263A>G and g. 276A>T were detected in the coding region of the GAL2 gene in Khuzestan native chickens. Three of the sequence variations recognized in the coding region were missense and the other mutation was nonsense. Missense mutations were g. 50T>C, g. 259C>G and g.263A>G that exchanged the amino acids 17, 26, and 27 of the GAL2 protein, respectively. The g. 50T>C substitution affected valine(GTT) to be replaced with alanine(GCT). The C-to-G mutation at 259 from the ATG-site caused arginine (CGG) to be subsituted with glycine (GGG). The g. 263A>G substitution changed aspartic acid (GAC) to glycine (GGC) amino acid. The nonsense mutation discovered in this study was an A-to-T substitution at 276 from the ATG-site. This mutation caused the cysteine (TGT) at the 31st amino acid of the GAL2 protein to be replaced with the stop codon (TGA).

Table 3. Sequence variation of part of the coding region of GAL2 gene in Khuzestan native chickens

Position based on AY621317.1 accession No.	Position based on A of the ATG-translation initiation codon	Mutation base	Genomic region	Mutation name
4868	-85	A>C	Upstream	g. -85A>C
5002	50	C>T	Exon 1	g. 50T>C
5111	159	G>T	Intron 1	g. 159G>T
5116	164	G>T	Intron 1	g. 164G>T
5211	259	C>G	Exon 2	g. 259C>G
5215	263	A>G	Exon 2	g.263A>G
5228	276	A>T	Exon 2	g. 276A>T
5345	393	G>T	Intron 2	g. 393G>T
5351	399	A>G	Intron 2	g. 399A>G
5417	465	A>G	Intron 2	g. 465A>G
5504	552	C>T	Intron 2	g. 552C>T
5553	601	A>G	Intron 2	g. 601A>G
5700	748	C>T	Intron 2	g. 748C.T
5759	807	C>T	Intron 2	g. 807C>T
5952	1000	C>T	Intron 2	g. 1000C>T

Discussion

This is the first report of sequence variations in GAL1 and GAL2 genes in Khuzestan native chickens. In about 2.4 kb of the genomic regions of these genes, we observed considerable genetic mutations. Many mutations were found in the 5' flanking region of GAL1 gene that includes the promoter region and is involved in starting the transcription of this gene. Thus, it is possible that

these variations may be disorder the function of genomic element. Therefore, genetic variations in promoter sequences of GAL1 gene are a perfect option for undertaking association studies and large-scale analyses in Khuzestan chickens. Consequently, we can suggest that sequence variations within the GAL1 gene promoter region may be causing phenotypic variation and the susceptibility to diseases in Khuzestan native chickens. Moreover, the nucleotide sequence analysis indicated that GAL2 had some intronic and exonic mutations. Although mutations in intronic regions did not affect the structure of protein molecules, they can provide excellent molecular markers for statistical association studies and genetic selection in animal breeding programs towards an increased immune response to diseases (Singh U. et al., 2014). In the coding region of GAL2 gene, some nonsynonymous mutations were observed that exchanged amino acids, resulting in biological and physiological changes that may be a cause of variation between individuals. A nonsense mutation was discovered inside missense mutations. Although both types of nonsynonymous mutations altered the amino acid sequence of the GAL2 protein, the nonsense g. 276A>T mutation changed a codon (31st amino acid) to a premature stop codon, resulting in the truncation of the GAL2 protein.

In this study, in 2.4 kb of the genomic regions belonging to GAL cluster genes, a total of 37 point mutations were discovered with the mutation rate of 15.4 SNPs/kb. This mutation rate was much higher than the 5 SNPs/kb across the whole chicken genome(Wong et al., 2004) and slightly more than the one previously reported for these genomic regions (13.2 SNPs/kb) in other chicken populations (Hasenstein et al., 2006). The high genetic variation in beta-defensin genes found in our study and other studies (Hasenstein et al., 2006), have a role in the innate immune response to Gram-negative and Gram-positive bacteria (Higgs et al., 2005; Sugiarto and Yu, 2004)and some viruses(Huang et al., 2013), increase resistance by enhancing the recruitment of macrophages, granulocytes, and lymphocytes to the infected tissues(Bar-Shira and Friedman, 2006), increase the expression in various tissues (Jang et al., 2015; Xiao et al., 2004), and genetic associations with immune response(Hasenstein and Lamont, 2007; Hasenstein et al., 2006) and bacterial loads (van Dijk et al., 2008), making the sequence variations of GAL1 and GAL2 genes in Khuzestan native chicken populations promising for marker-assisted genetic selection studies.

The results of the present study revealed that the genetic background of Khuzestan native chickens is highly polymorphic. This result is notable because the high genetic variation considered in our study was located in regions whose association with innate immune response to bacteria and viruses (Aguilar-Jimenez et al., 2013; Higgs et al., 2005; Huang et al., 2013; Sugiarto and Yu, 2004) and role in increasing resistance to diseases(Bar-Shira and Friedman, 2006) had previously been reported. It is essential to consider local poultry resources for further research because these populations can be a valuable gene pool or major supply of genes as an indigenous poultry population resistant to the local harsh climate. Moreover, Khuzestan native chicken populations can be a valuable gene pool or major supply of genes as indigenous chickens to consider in genetic selection and animal breeding programs especially for disease resistance in poultry industry.

The heritability of some traits belonging to resistance to diseases is varied between 0.06 and 0.32 (Berthelot et al., 1998; Girard-Santosuosso et al., 1998). These estimates show that there is a genetic basis for the resistance to diseases, and thus using the breeding value calculated based on phenotype and pedigree can improve the phenotype. However, it is essential to employ genomic selection in breeding programs for resistance to diseases in poultry industry. Since the chicken genome was sequenced (International Chicken Genome Sequencing, 2004), there is a chance to evaluate local chickens reared in different areas to use their indigenous potential in poultry industry breeding programs. The beta-defensin genes, especially the regions investigated in our study, can be considered in further studies as candidate genes.

Conclusion

Utilizing native animals in breeding programs has been considered in some countries. In this regard, studying genetic resources, particularly genomic regions that are associated with diseases and their control in native animals, is very important. In the present study, genetic variations of GAL1 and GAL2 genes were studied in Khuzestan native chickens reared in villages. In summary, results revealed that the sequence of some genes that play a central role in response to pathogens had mutations present in promoter, intronic, and exonic regions. Sequence variations considered in GAL1 and GAL2 can potentially be employed in marker-assisted selection programs to enhance the response to pathogens. It is therefore possible to use these genes in poultry breeding programs in order to significantly reduce the amount of drug costs and prevent the decline in production. Furthermore, breeding disease-resistant poultry will provide healthy food for consumers with a little amount of antibiotics and pathogens such as bacteria and viruses.

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