Association between Single nucleotide polymorphisms in Gallinacin genes and resistance to Marek's disease in White Leghorn chicken

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Abstract: Gallinacins are antimicrobial peptides that play a significant role in innate immunity in chicken. The aim of this study was to determine the relationship between candidate genes of innate immunity and resistance to Marek's disease and to predict whether the amino acids substitutions lead to produce new phenotypes. We used in current study two inbred lines of White Leghorn chickens, line 6 which selected for resistant to Marek's disease and line 7 which selected to susceptible to Marek's disease from ADOL, ARS,USDA. We examined Gal-1 and Gal-2 in current study by sequenced a 1.38 kb in two directions from two inbred lines (6 and 7). A total of 6 SNPs were identified within the sequenced regions. This equates to an SNP rate of 4.34 SNPs/kb, nearly to the previously reported 5 SNPs/kb across the entire chicken genome. The current study showed that the gallinacin genes are polymorphic because there are many single nucleotide polymorphisms (SNPs) in both inbred lines of White Leghorn chickens and some of these SNPs are nonsynonymous and others are synonymous and some of them are located in intronic region and the rest are in exonic region. All identified SNPs were intronic; except for Gal-1 was exonic resulting in amino acids changes which have a non-synonymous SNP resulting in amino acids alterations of asparagine to serine, histidine to tyrosine and tyrosine to serine, respectively. From SIFT (Sorting Intolerant from tolerant) program which used to predict whether an amino acids substitutions can affect protein function resulting in phenotypic effect , that is may be made the inbred line 7 of White Leghorn chickens are susceptible to Marek's disease rather than line 6. We are concluded that a new chromosomal region with effects on the response to Marek's disease in chickens was characterized in this study. Within this region, the SNPs in the gallinacin candidate genes could potentially be used in a marker assisted selection program to enhance the response to Marek's disease. Analysis of the gallinacin genes in the protective pathways of disease resistance has also opened the possibilities for therapeutic strategies using endogenous antimicrobial peptides. [Journal of American Science. 2010;6(11):109-114]. (ISSN: 1545-1003).

Keywords: single nucleotide polymorphisms, Gallinacin, genes, Marek's disease, resistance

1. Introduction

Global production of chickens has experienced massive change and growth over the past 50 years. The commercial broiler and layer markets produce more than 50 billion birds annually to meet current worldwide consumer demands of more than 74 metric tons of meat and more than 66 million metric tons of eggs (Muir *et al.*, 2008).

In fact, poultry has become the leading meat consumed in the United States and most other countries and is the most dynamic animal commodity in the world; production has increased by 436% since 1970, more than 2.3 times and 7.5 times the corresponding growth in swine and beef, respectively (http//faostat.fao.org). Unfortunately, the poultry industry continues to be confronted with new and emerging infectious diseases such as Newcastle disease, avian leucosis, avian influenza and Marek's disease that can led to significant economic losses.

Marek's disease (MD) is a lymphoproliferative disease, caused by a member of the herpesvirus family, that is estimated to cost the poultry industry nearly $1 billion annually (Purchase, 1985). Diseased chickens infected by the Marek's disease virus (MDV), the causative pathogen, commonly exhibit paralysis, blindness, and visible lymphoid tumors that result in condemnation of the birds. Although vaccination programs have effectively reduced the incidence of MD, there is evidence that current vaccines do not protect well against some highly pathogenic MDV strains that have emerged in recent years (Witter and Hunt, 1993). Also, MD vaccines control rather than eliminate losses from MD because they do not block MDV infection, thus as a result, MDV is ubiquitous on poultry farms, and all chickens are exposed to the pathogenic agent at 1 day of age (Vallejo *et al.*, 1997).

All these factors point to the need to complement vaccinal protection with alternative methods such as genetic resistance (Spencer *et al.* 1974; Gavora and Spencer, 1979). And even if a

specific disease has been controlled through vaccination, genetic resistance is of value because it represents a safeguard against heavy losses in the case of disease outbreaks (Vallejo *et al.*, 1997).

Genetic resistance to MD has been known for more than 60 years (Calnek, 1985). Genetic resistance is a complex trait controlled by many genes though genetic selection for high levels of resistance can be obtained within relatively few generations (Cole, 1968), this is because of selection for certain MHC haplotypes, something that would not be done now to maintain biodiversity. The development of effective vaccines in the late 1960s, however, greatly reduced interest in the genetic control of MD. Ironically, genetically resistant lines were shown to have greater vaccinal immunity and higher egg production than susceptible lines (Von Krosigk *et al.*, 1972; Spencer *et al.* 1974; Gavora and Spencer, 1979).

One such class of genes that may play a role in resistance to Marek's disease are gallinacin genes, one family of antimicrobial peptides (AMP). Antimicrobial peptides (AMP) are relatively small molecules that are less than 100 amino acids in length and have a broad spectrum of antimicrobial activity (Ma *et al.*, 2007). Defensins are a type of AMP characterized by the presence of a conserved cysteine (Cys)-rich defensin motif. The three defensin subfamilies (α, β and θ defensins) found in humans and mammals, only β–defensins have been found in birds (Sugiarto and Yu, 2004; Satchell *et al.*, 2003; Bensch *et al.*, 1995 and Higgs *et al.*, 2005).

These Gals are widely expressed across most tissues, including those of the digestive system, respiratory system, genitourinary system, and several other anatomical areas in the chicken (Ma *et al.*, 2007). Further, different Gals are expressed in different tissues (Higgs *et al.*, 2005; Harwing *et al.*, 1994 and Lynn *et al.*, 2004).

The main objectives of this study is

1. To identify and analyze new candidate genes for their association with resistance to Marek's disease in the inbred White Leghorn Lines 6 subline 3 (6_3) and 7 subline 2 (7_2).
2. To predict whether an amino acid substitution in a protein will have a phenotypic effect.

2. Material and Methods

This study was carried out, at the Avian Disease and Oncology laboratory (ADOL), Agricultural Research Service (ARS), United States Department of Agriculture (USDA), USA and Cell Biology Department, National Research Center of Egypt. The inbred White Leghorn Lines 6 subline 3 (6_3) and 7 subline 2 (7_2) had been taken to be used in current study, differ greatly in MD susceptibility (6_3 is resistant and 7_2 is highly susceptible; Crittenden, 1975; Pazderka et al., 1975).

1. DNA isolation, PCR

Genomic DNA was prepared from chicken erythrocytes by using QIAgen DNA purification kit. To characterize the 3'-untranslated region of each gene, a pair of primers **(Table 1)** was developed using FastPCR, based on the published chicken genome assembly. PCRs were performed using 25-µl reaction mixture volumes that contain 25 ng of chicken genomic DNA, 0.8 µM of each primer,200µM of each deoxynucleoside triphosphate, 1 unit of *Taq* DNA polymerase , 2.5 µl of 10x PCR buffer, and 1.5mM $MgCl_2$.

The following cycling conditions were used:

1. An initial denaturation step at 94 ^{0}C for 3 min, followed by 35 cycles at
2. 94 ^{0}C for 1 min,
3. at the optimal annealing temperature for 1 min, and at
4. 72 ^{0}C for 1 min and
5. Final extension at 72 ^{0}C for 5 min. The PCR products were separated by electrophoresis through 1.5% gel.

2. The sequencing

The PCR products were purified using Sephedex-G, An ABI3100 DNA analyzer (Applied Biosystems, Foster City, CA) was used for direct sequence using nucleotide dye terminators. PCR products were sequenced at Avian Disease and Oncology Laboratory (ADOL), ARS, USDA.

3. Sequencing analysis

Sequencing alignment was achieved using Nucleotide-nucleotide BLAST (blastn) software in htt://www.ncbi.nlm.nih.gov/blast/ and CLASTALW 2.0.12**.** To detect the SNPs in inbred White Leghorn lines using Sequencher program version 4.8, also, to predict whether an amino acid substitution in a protein will have a phenotypic effect using Sorting Intolerant from Tolerant (SIFT) program http://sift.jcvi.org/www/SIFT_aligned_seqs_submit.html.

3. Results

1. Sequence variation

1.1. Gallinacin-1

Single nucleotide polymorphism in gallinacin 1 in line 6 (resistant to Marek's disease) and line 7 (susceptible to Marek's disease) is showed in Fig (3). Primers designed from gallinacin 1 genomic DNA amplified a 872 bp fragment of the gene with SNPs of A-to-G, C-to-T and A-to-C within the exonic sequence in line 6 (resistant to MD) and line 7 (susceptible to MD), respectively. At positions 110,260,751, 110,260,716 and 110,260,781 of the chicken genome assembly the nucleotides are G, T and C, respectively. These nonsynonymous SNPs produced an amino acid change from asparagine to serine, histidine to tyrosine and tyrosine to serine, respectively.

Single nucleotide polymorphism in gallinacin 2 in line 6 (resistant to Marek's disease) and line 7 (susceptible to Marek's disease) is noticed in Fig (4). For gallinacin 2 , a 553 bp product that contain two substitutions in an intron A- to G, A-to-G and A-to-G in line 6 (resistant to MD) and line 7 (susceptible to MD), respectively. At position 110,258,387,110,258,196 and 110,258, 137 of the chicken genome assembly the nucleotides are A, A and G respectively. This SNP is a synonymous SNP and it doesn't change amino acid.

1.2. Gallinacin-2

Table. 1. Primer sequence of Gal-1 and Gal-2 .

Gene	Primer sequence (Forward/reverse)	PCR product size bp	Annealing temperature	Accession Number
Gal -1	5'-ACTGCAGGCCCATGGTGGGATGTC-3' 5'-TGTTAGACTGAGATCCATGGGAC-3'	827	58	HM136609 HM136612
Gal-2	5'-GCTGCTGAGGCTTTGCTGTAGC-3' 5'-ATGGCCATAGATGCCAGCCAC-3'	553	58	HM136610 HM136611

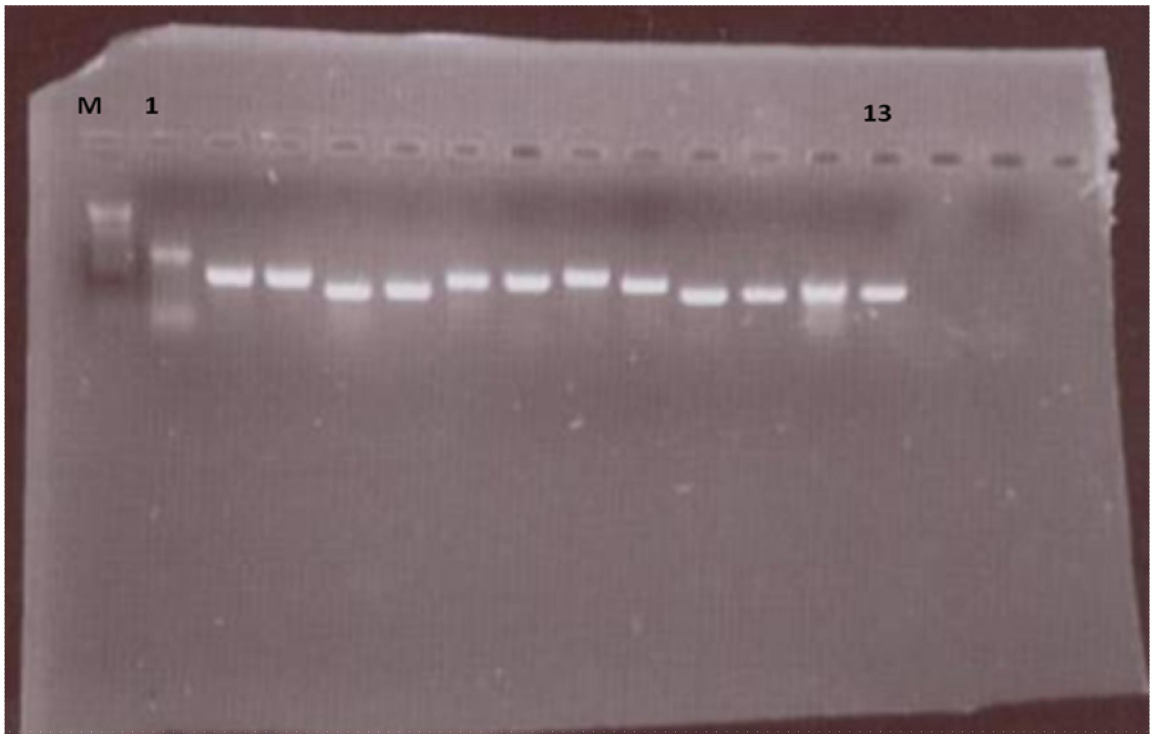

Fig.1. Amplified fragment of gallinacin genes (1-13) in inbred White Leghorn line 6 sub line 3. Lane M, DNA molecular weight marker. Lane 1-13, Gal-1- Gal-13.

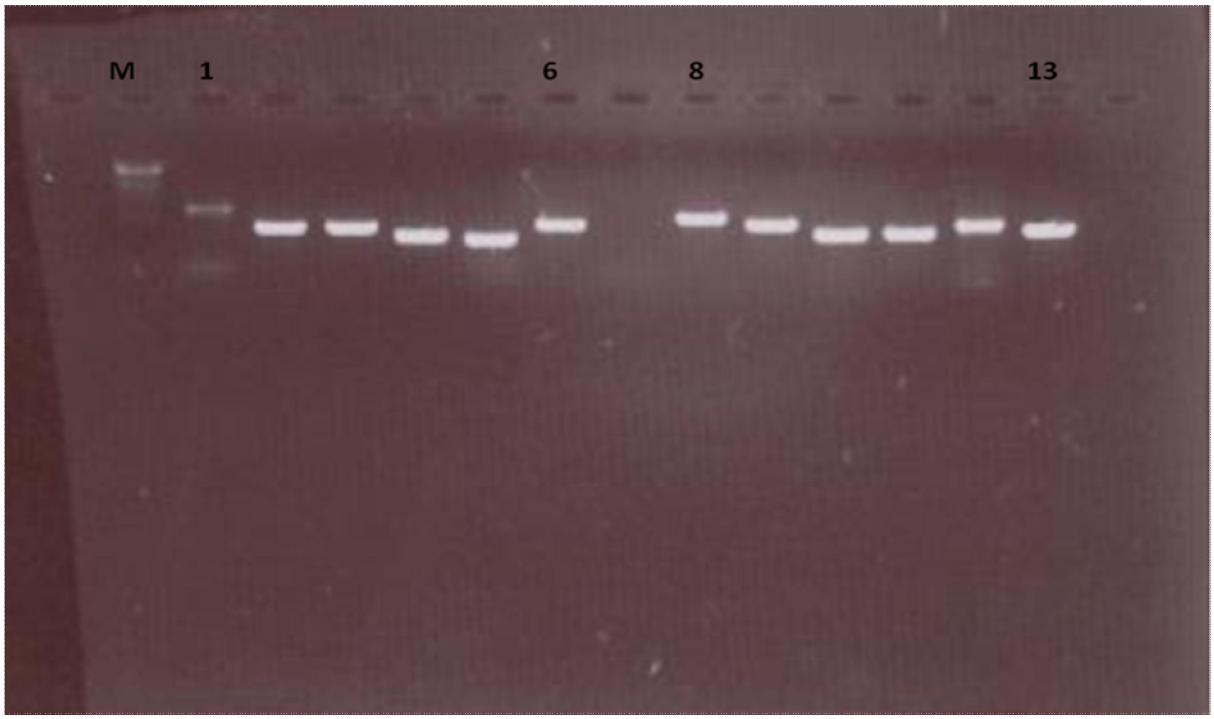

Fig.2. Amplified fragment of gallinacin genes (1-6)(8-13) in inbred White Leghorn line 6 sub line 3. Lane M, DNA molecular weight marker. Lane 1-6 , Gal-1 –Gal-6. Lane 8-13, Gal-8 – Gal-13.

4. Discussion

SNP detection and its rate

In total, 1.38 kb was sequenced in two directions from two inbred lines (6 and 7). A total of 6 SNPs were identified within the sequenced regions. This equates to an SNP rate of 4.34 SNPs/kb, nearly to the previously reported 5 SNPs/kb across the entire chicken genome (Wong *et al.*, 2004).

All identified SNPs were intronic, except for Gal-1 was exonic resulting in an amino acids changes which have a non-synonymous SNP resulting in amino acids changes of asparagine to serine, histidine to tyrosine and tyrosine to serine, respectively.

Non-synonymous SNP are of interest due to their potential effect on protein expression and, ultimately have minimal effects on genes expression (exceptions might be those nucleotides that are important in DNA–protein interactions in the promoter and the genomic regions or those nucleotides that are involved in RNA stability) and both synonymous and non-synonymous SNP are excellent genetic markers for mapping studies **(Emara and Kim, 2003).**

SNPs location

The current study showed that the gallinacin genes are polymorphic because there are many single nucleotide polymorphisms (SNPs) in both inbred lines of White Leghorn chickens and some of these SNPs are nonsynonymous and others are synonymous and some of them are located in intronic region and the rest are in exonic region.

Intronic SNPs

There were many intronic SNPs are located in non-coding region in gallinacin genes specifically for gal-2, 3 SNPs. Intronic SNPs, while not the causal mutations, can provide excellent markers for genetic selection for an increased immune response to Marek's disease.

Exonic SNPs

In a gallinacin 1 there were three nonsynonymous substitutions A-to-G, C-to-T and A-to-C within the exonic sequence in line 6 (resistant to MD) and line 7 (susceptible to MD), respectively. And these alterations lead to protein modification through changes of asparagine to serine, histidine to tyrosine and tyrosine to serine in lines 6 and 7, respectively.

From SIFT (Sorting Intolerant from tolerant) program which used to predict whether an amino acids substitutions can affect protein function resulting in phenotypic effect , that is may be made the inbred line 7 of White Leghorn chickens are susceptible to Marek's disease rather than line 6.

Most genetic variation is considered neutral but single base changes in and around a gene can affect its expression or the function of its protein products (Collins *et al.,* 1997 and Risch and Merikanges, 1996). A nonsynonymous or missense variant is a single base change in a coding region that causes an amino acid change in the corresponding protein.

If a nonsynonymous variant alters protein function, the change can have drastic phenotypic consequences. Most alterations are deleterious and so are eventually eliminated through purified selection. However, beneficial mutations can sweep through the population and become fixed, thus contributing to species differentiation.

It was observed that disease-causing Amino Acid Substitutions (AASs) had common structural features that distinguished them from neutral substitutions, suggesting that structure could also be used for prediction (Sunyaev *et al*., 2000 and Wang and Moult, 2001).

The gallinacin genes are clustered within an 86-kb distance on the 3q3.5-q3.7 chromosome (Xiao *et al.,* 2004). The location of molecular markers within this cluster could be useful for marker assisted genetic selection and positional cloning works (Hasenstein *et al.,* 2006).

Bar-Shira et al. (2006) hypothesized that innate effector mechanisms such as gallinacin enable immune protection during the first week after hatching until functional maturation of the adaptive immune system occurs. They showed that mRNA levels of *Gal1* and *Gal2* decreased relative to the day of hatching throughout the first week of life and then increased again during the second week.

5. Conclusion:

We concluded that a new chromosomal region with effects on the response to *Marek's disease* in chickens was characterized in this study. Within this region, the SNPs in the gallinacin candidate genes could potentially be used in a marker assisted selection program to enhance the response to Marek's disease. Analysis of the gallinacin genes in the protective pathways of disease resistance has also opened the possibilities for therapeutic strategies using endogenous antimicrobial peptides**.**

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