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308 Lasani Town, Sargodha Road, Faisalabad - Pakistan  
Mob: +92 300 3008585, Fax: +92 41 8815544  
E-mail: editorijps@gmail.com

## Association Between Candidate Genes of Innate Immunity, Gallinacin Genes and Resistance to Marek's Disease in Chicken

H.A. Yacoub<sup>1,2</sup>, A. Galal<sup>3</sup>, M.M. Fathi<sup>3,4</sup>, S.A. El Fiky<sup>1</sup> and H.A.I. Ramadan<sup>1,2</sup>

<sup>1</sup>Department of Cell Biology, National Research Center, Dokki, Giza, Egypt

<sup>2</sup>Department of Biological Sciences, Faculty of Science, King Abdul Aziz University, Jeddah, Kingdom of Saudi Arabia

<sup>3</sup>Department of Poultry Production, Faculty of Agriculture, Ain Shams University, Cairo, Egypt

<sup>4</sup>Department of Production and Breeding, Faculty of Agriculture and Veterinary Medicine, Qassim Univeristy, Qassim, Kingdom of Saudi Arabia

**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 gallinacin genes 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 White Leghorn Lines 6 and 7. We examined gallinacins genes (3-5 and 10) by sequenced a 2.29 kb in two directions from two inbred lines (6 and 7). A total of 10 SNPs were identified within the sequenced regions. This equates to an SNP rate of 4.36 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 (SNPs) in both inbred lines of White Leghorn chickens and some of these SNPs are nonsynonymous and others are synonymous. 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.

**Key words:** Innate immunity, gallinacin, genes, Marek's disease, resistance

### 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 million 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 (<http://faostat.fao.org> and Satchell *et al.*, 2003). 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).

Table 1: Primer sequence of Gal-1 - Gal-5 and Gal-10

Gene	Primer sequence (Forward/reverse)	PCR product size bp	Accession number
Gal-3	5'-CACGGTCATACCATGGGAGACTGC-3'	567	HM136603
	5'-GTTTGTGATGTTGCTGCAAAGAC-3'		
Gal-4	5'-AGACTTCTGGAGATCCTGTGC-3'	440	HM136605
	5'-GTGGTAAGTGTCTCCAGGTG-3'		
Gal-5	5'-CATAGTGTTAATGCAAGCTGCCAC-3'	808	HM136607
	5'-ATGCTGCTGGGCTGCAGCAATCC-3'		
Gal-10	5'-GGTTGTGTGCAGACCTACACCC-3'	477	HM179988
	5'-CTGAGCATCCAAGATCCCGTG-3'		

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).

The main objectives of this study is:

- To screen candidate (gallinacin genes) in the inbred White Leghorn Lines 6 subline 3 (63) and 7 subline 2 (72), which are Marek's disease resistant and susceptible, respectively.
- To predict whether an amino acid substitution in a protein will have a phenotypic effect on Marek's disease.

### MATERIALS 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 (63) and 7 subline 2 (72) had been taken to be used in current study, differ greatly in MD susceptibility (63 is resistant and 72 is highly susceptible (Pazderka *et al.*, 1975; Muir *et al.*, 2008).

**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, 200 µM of each deoxynucleoside triphosphate, 1 unit of Taq DNA polymerase, 2.5 µl of 10x PCR buffer and 1.5 mM MgCl<sub>2</sub>. The PCR cycle profile was 94°C for 3 min before the first cycle, then 94°C for 1 min, 58°C for 1 min and 72°C for 1 min for 35 cycles. After the last cycle, the PCR mixture was incubated for a further 5 min at 72°C. The reaction products (5 µl each) were used for electrophoresis with an appropriate size marker on 1.5% agarose in 1X-Tris acetate buffer (TAE). After electrophoresis the gels were stained with ethidium bromide and were examined with UV lamp at a wave length 312 nm to verify amplification

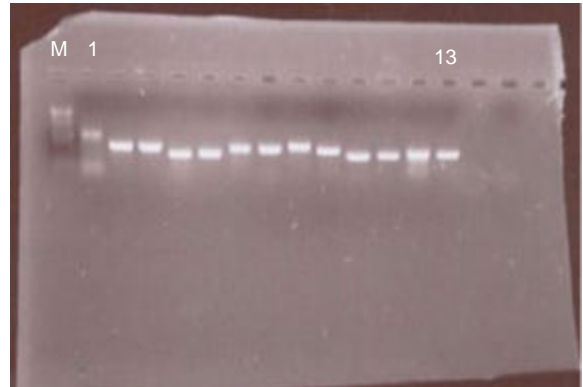


Fig. 1: Amplified fragment of gallinacin genes (1-13) in inbred White Leghorn line 6 sub line 3. Lane M, DNA molecular weight marker. Lane 1-13, Gal-1-Gal-13

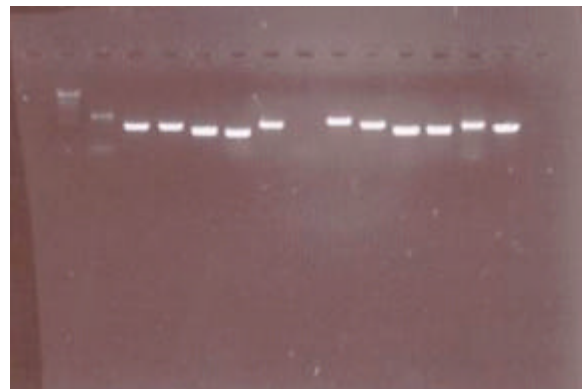


Fig. 2: Amplified fragment of gallinacin genes (1-6) (8-13) in inbred White Leghorn line 6 sub line 3. Lane M, DNA molecular weight marker. Lane 1-6, Gal-1 - Gal-6. Lane 8-13, Gal-8 - Gal-13

of the gallinacin genes fragments. The PCR products were purified using QIAquick PCR purification kit (Qiagen, Inc.) and the resulting purified products were used in the subsequent sequencing reactions. Sequencing was performed on an Applied Biosystems 3100 genetic analyzer (Applied Biosystem) using BigDye terminator cycle sequencing ready reaction mixture according to manufacturer's instructions (Applied Biosystems).

**Sequencing analysis:** Sequencing alignment was achieved using Nucleotide-nucleotide BLAST (blastn) software in (<http://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](http://sift.jcvi.org/www/SIFT_aligned_seqs_submit.html)).

**RESULTS AND DISCUSSION**

**Sequence variation:** There were many intronic SNPs are located in non-coding region in gallinacin genes specifically for gal-3 and gal-5, 6 SNPs and 3 SNPs, respectively. Intronic SNPs, while not the causal mutations, can provide excellent markers for genetic selection for an increased immune response to Marek's disease. 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 located in intronic region and the rest are in exonic region.

**Gallinacin-3:** Single nucleotide polymorphism in gallinacin 3 in line 6 and line 7 is mentioned in Fig. 3. A 567 bp product amplified from gallinacin 3 genomic DNA, had many substitution SNPs consisted of A-to-C, T-to-C, G-to-T, G-to-A, G-to-A and C-to-T in line 6 and line 7, respectively. This gene has the same nucleotide in both two line of chicken which is (T) but it differ in chicken genome assembly which is (G) at position of 110,264,990.

**Gallinacin-4:** Single nucleotide polymorphism in gallinacin 4 in line 6 and line 7 is showed in Fig. 4. An SNP in the gallinacin 4 was found to be within an exonic region and consisted of a A-to-G substitution in a 440 bp PCR product in line 6 and line 7, respectively. This SNP is a synonymous SNP and it doesn't change amino acid however it was found in an exonic region.



Fig. 3: CLUSTAL 2.0.12 multiple sequence alignment of gallinacin 3 between line 6(resistance line) and line 7(susceptible line)

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line-6-   ACGGATTGAGAAGAGAGGGAGAAGTGGGAGAATATCGTATCTGCACAGTCTCCCTTTTC 60
line-7-   ACGGATTGAGAAGAGAGGGAGAAGTGGGAGAATATCGTATCTGCACAGTCTCCCTTTTC 60
          *****

line-6-   TTTTCCTTCTTTTTTCAATTTTCTTTTCTCTTTTTTAAATACTGCAGGCTTTTCCCGT 120
line-7-   TTTTCCTTCTTTTTTCAATTTTCTTTTCTCTTTTTTAAATACTGCAGGCTTTTCCCGT 120
          *****

line-6-   TCTCCAGATATCATATGCAATGTGGATATCGCGGGACCTTCTGCACCCCTGGGAATGCC 180
line-7-   TCTCCAGATATCATATGCAATGTGGATATCGCGGGACCTTCTGCACCCCTGGGAATGCC 180
          *****

line-6-   TTATGGGAATGCTTACCTGGGGCTATGCCGTCAGTATTCTTCTGTAGATGGAAGATT 240
line-7-   TTATGGGAATGCTTACCTGGGGCTATGCCGTCAGTATTCTTCTGTAGATGGAAGATT 240
          *****

line-6-   AAGACTTGACTATGGCTAAACTGACTTCCAGATTTAAGTCATATGGTGGGTTTCCCTT 300
line-7-   AAGACTTGACTATGGCTAAACTGACTTCCAGATTTAAGTCATATGGTGGGTTTCCCTT 300
          *****

line-6-   CACTTACGTGTGAACCTGTACTCCTCTCTTTGATAGATAGAAGAAAAATGAAGTCCCC 360
line-7-   CACTTACGTGTGAACCTGTACTCCTCTCTTTGATAGATAGAAGAAAAATGAAGTCCCC 360
          *****

line-6-   AAAATCTAAATGGGTATATAGAGAGAAAAAGAGGGGGGGAGGG 405
line-7-   AAAAT----- 365
          *****

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Fig. 4: CLUSTAL 2.0.12 multiple sequence alignment of gallinacin 4 between line 6 (resistance line) and line 7(susceptible line)

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line-6-   -AGGTGTC-TCCCTCCACCCTAATGTTGGCAGCCAGCCACCCTGTAGAGAGCATGG 58
line-7-   CGAGTGTCTCCTTTCCACCCTTAGCGCTGGCAGCCAGTCTCCACCCTGTAGAGAGCATGG 60
          *****

line-6-   GGACTCTTCCAGGCTTCCACCAGCCCCAGAACCCTAGTTTCTGAGAGCAGTCCCAAAGGGA 118
line-7-   GGACTCTTCCAGGCTTCCACCAGCCCCAGAACCCTAGTTTCTGAGAGCAGTCCCAAAGGGA 120
          *****

line-6-   GATGGGCATTTTAACTGAGGGTCTGGCCTCATAGAGTTGGGATGAACACTGCCACACCT 178
line-7-   GATGGGCATTTTAACTGAGGGTCTGGCCTCATAGGGTTGGGATGAACACTGCCACACCT 180
          *****

line-6-   TTCTCCACAGCCGATGGTATTCCTGATGGCCTCTGATGTCCCTTCCAGTTCATCTCCA 238
line-7-   TTCTCCACAGCCGATGGTATTCCTGATGGCCTCTGATGTCCCTTCCAGTTCATCTCCA 240
          *****

line-6-   TGCAGAGCATCGAAGCCACCACCTACCTTTGAGTGCACCAGAGGAACAATCACCCTGG 298
line-7-   TGCAGAGCATCGAAGCCACCACCTACCTTTGAGTGCACCAGAGGAACAATCACCCTGG 300
          *****

line-6-   GAGCGGTGGTGACCCCTGGACAACCAGACCTCAGGCCCGTGAGAACCCCTCTGTCAAAAT 358
line-7-   GAGCGGTGGTGACCCCTGGACAACCAGACCTCAGGCCCGTGAGAACCCCTCTGTCAAAAT 357
          *****

line-6-   AAAGGTGGCAGCTTGATTTAACACTATGATA 389
line-7-   AAAG----- 361
          *****

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Fig. 5: CLUSTAL 2.0.12 Multiple sequence alignment of gallinacin 5 between line 6(resistance line) and line 7(susceptible line)

**Gallinacin-5:** Single nucleotide polymorphism in gallinacin 5 in line 6 and line 7 is illustrated in Fig. 5. For gallinacin 5, a 808 bp product had several substitution SNPs within an intron, which is consisted of A-to-G, C-to-T and A-to-T in line 6 (resistant to MD) and line 7 (susceptible to MD), respectively.

**Gallinacin-10:** For a gallinacin 10, 477 bp PCR products, has no SNP in both lines of chicken in our sequencing Data as showed in Fig. 6.

**SNP detection and its rate:** In total, 2.29 kb was sequenced in two directions from two inbred lines



Fig. 6: CLUSTAL 2.0.12 Multiple sequence alignment of gallinacin 10 between line 6(resistance line) and line 7(susceptible line)

(6 and 7). A total of 10 SNPs were identified within the sequenced regions. This equates to an SNP rate of 4.36 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-4, we found only one synonymous substitution A-to-G in exonic region but this SNP doesn't affect protein function and doesn't lead to change the phenotype according to SIFT (Sorting Intolerant from tolerant) program, in spite of its location in coding region. 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. 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). The current study suggested that this is a clear evidence that the inbred lines of White Leghorn chicken which used for selection to resistant to Marek's disease differ than other types of chicken, this can be illustrated from

SNP in Gallinacin 3, both inbred lines 6 and 7 have the same base (T) while the substitution is a (G) in genome assembly at position 110,264,990 of the chromosome 3, this SNP is located in non-coding region which means that it can be used to diverse both lines from other chicken and it may be possible to consider it as a biodiversity maker. 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 (Calnek and Witter, 1997; 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; Wang and Moul, 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 (Hasenstien *et al.*, 2006). Bar-Shira and Friedman (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.

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.

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