ISSN 1682-8356
ansinet.org/ijps

INTERNATIONAL JOURNAL OF

POULTRY SCIENCE

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

OPEN ACCESS International Journal of Poultry Science

ISSN: 1682-8356
DOI: 10.3923/ijps.2017.336.343

Research Article
Haplotype Structure and DNA Sequence Variation of the Liver Expressed Antimicrobial Peptide-2 (*chLEAP-2*) Gene in Chickens challenged with *Eimeria maxima*

[1]A.M.J.B. Adikari, [2]J. Xu, [3]S. Casterlow, [4]H. Li, [2]E.R. Gilbert, [2]A.P. McElroy, [5]D.A. Emmerson, [2]R.A. Dalloul, [2]E.A. Wong and [2]E.J. Smith

[1]Department of Animal and Food Sciences, Faculty of Agriculture, Rajarata University of Sri Lanka, 50000 Anuradhapura, Sri Lanka
[2]Department of Animal and Poultry Sciences, Virginia Tech, Blacksburg, 24061 VA, USA
[3]Department of Biomedical and Veterinary Sciences, Virginia Tech, Blacksburg, 24061 VA, USA
[4]Shanxi Agriculture University, Shanxi Province, China
[5]Aviagen Inc., Cummings Research Park, Huntsville, 35805 AL, USA

Abstract

Objective: The aim of this study was to screen the *chLEAP-2* gene for DNA sequence variation and to evaluate the relationships among its haplotypes (based on haplogroups), expression levels, weight gain and lesion score in two chicken lines challenged with *Eimeria maxima*. **Methodology:** A total DNA sequence of 4.6 kb including the *chLEAP-2* gene was screened by re-sequencing of individual amplicons. Sixteen SNPs, including seven each in the promoter and introns and two in exons, were identified. **Results:** One of the exonic single-nucleotide polymorphism (SNPs) was non-synonymous, involving a cysteine to tyrosine codon change. About 25% of the SNPs were in Hardy Weinberg equilibrium. Linkage disequilibrium (D´) among the SNPs ranged from 0.02-1.00. The haplotypes observed from the 16 SNPs were assembled into 5 haplogroups. The estimated frequencies of the haplogroups ranged from 0.17-0.23 in the combined chicken lines. Although not significant ($p \geq 0.05$), the *chLEAP-2* gene expression varied among haplogroups. Differences among haplogroups for lesion score and weight gain were consistent, but not statistically significant ($p \geq 0.05$). However, Hap4 appeared to be the haplogroup least susceptible to coccidiosis. At a minimum, the data do not support an association between *chLEAP-2* DNA sequence variation and symptoms of coccidiosis such as weight gain depression and lesion score. **Conclusion:** Therefore, earlier reports of differences between resistant and susceptible lines in *chLEAP-2* expression may be due to trans-acting factors. The genomic results reported here provide resources for testing the trans-expression control theory and will be useful for future genotype:phenotype evaluation studies between *chLEAP-2* and other traits in the chicken.

Key words: Chicken, liver expressed antimicrobial peptide-2, coccidiosis, haplogroups, *Eimeria maxima*

Received: June 25, 2017 Accepted: July 23, 2017 Published: August 15, 2017

Citation: A.M.J.B. Adikari, J. Xu, S. Casterlow, H. Li, E.R. Gilbert, A.P. McElroy, D.A. Emmerson, R.A. Dalloul, E.A. Wong and E.J. Smith, 2017. Haplotype structure and DNA sequence variation of the liver expressed antimicrobial peptide-2 (*chLEAP-2*) gene in chickens challenged with *Eimeria maxima*. Int. J. Poult. Sci., 16: 336-343.

Corresponding Author: A.M.J.B. Adikari, Department of Animal and Food Sciences, Faculty of Agriculture, Rajarata University of Sri Lanka, 50000 Anuradhapura, Sri Lanka Tel: 0094-718262001, Fax: 0094-252221614

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Coccidiosis is an intestinal disease that affects many animals, including birds. In poultry, it is a major cause of economic losses to the industry worldwide. Seven species of *Eimeria* are responsible for causing avian coccidiosis in chickens[1]. Infections by the parasites produce lesions as a result of damaged intestinal epithelia, leading to reduced bird performance. Infection of the mid small intestine caused by *Eimeria maxima* (*E. maxima*) is one of the most economically devastating diseases to the poultry industry. The gross clinical signs include lesions with a salmon pink exudate, thickened intestinal wall and haemorrhagic patches. Though its pathogenicity is considered medium, mortality can be as high as 20%[2]. Reducing the damage and associated losses caused by *E. maxima* thus remains a major goal of the poultry industry.

Biological molecules like host defense peptides (HDP) are essential components of the innate defense system with both antimicrobial and immunomodulatory properties[3,4]. Host defense peptides are synthesized in response to invasion and infection by microbes and may afford the host's capacity to resist *E. maxima* infection. The HDPs disrupt the membrane integrity of invading microbes or inhibit metabolic processes of the pathogen[5]. Two HDPs identified in vertebrates that are involved in the host defense mechanism are liver-expressed antimicrobial peptide LEAP-1 and LEAP-2 [2]. LEAP-1, the first discovered blood-derived HDP, is secreted from the liver and is involved in iron homeostasis[6]. LEAP-2, discovered shortly thereafter, is expressed in a number of tissues including the small intestine, lungs and kidney, with greatest expression in the liver[7].

The chicken *LEAP-2* gene (*chLEAP-2*) was originally described by Smith *et al.*[8] in a genome-wide expression profiling as a member of chicken immune related genes. The gene is located on chicken chromosome 13 and has three exons that together encode a 76-amino acid peptide[9]. The encoded peptide exhibits a classic pre/pro-peptide structure. The pro-LEAP-2 is secreted as a 53 amino acid pro-peptide, which is cleaved to a 40 amino acid mature peptide that contains two disulfide bonds[10]. The chicken LEAP-2 has been shown to kill enteric pathogens, though the mechanism remains unclear[11]. Chicken LEAP-2 also functions in preventing pathogenic microbes from interacting with the epithelial surface, thereby impeding microbial invasion of tissues[9].

In an earlier study, Casterlow *et al.*[12] reported that two genetically selected chicken lines (A and B) showed differential *chLEAP-2* gene expression in response to an *E. maxima* challenge with line A birds exhibiting higher resistance compared to line B. In the genome-wide expression analysis of the lines using DNA microarrays, they showed that though *chLEAP-2* was down-regulated by 20-fold in line A, the down-regulation in line B was on average much higher and ranged from 11- to 71-fold. Subsequent studies in layers and broilers have demonstrated that there is down-regulation of *chLEAP-2* following infection with not only *E. maxima* but also *E. praecox*, *E. acervulina* and *E. tenella*[13-16]. In the current study it is hypothesized that differences in the *chLEAP-2* gene expression in selected lines is associated with DNA sequence variation based on SNPs and haplotype frequencies. The objectives of the current study, therefore, were to screen the *chLEAP-2* gene for DNA sequence variation and to evaluate the relationships among its haplogroups, gene expression levels, lesion score (LS) and weight gain (WG).

MATERIALS AND METHODS

DNA sequence analyses: Blood samples of Aviagen's lines A and B chickens were used from the study of Casterlow *et al.*[12]. The selection history and performance characteristics of the two lines were described by Gilbert *et al.*[17] with the two lines exhibiting differential responses to *Eimeria* infection[12]. The genomic DNA from 82 birds (41 from Line A and 41 from Line B) were isolated using a standard salting out procedure[18]. The DNA sequence of the *chLEAP-2* gene (GenBank accession No: LOC414338) was used to design primers using primer 3[19]. The information for the primers including the sequences, annealing temperature and expected sizes of the PCR amplicons are presented in Table 1. The PCR amplification was performed in a final volume of 25 μL consisting of standard reagents including Taq DNA polymerase (Takara Bio, Inc., Japan), 200 μM dNTPs and 2 mM $MgCl_2$. Following PCR, each

Table 1: Primer sequences, the expected sizes of amplicons and PCR characteristics for chLEAP-2 gene

Primer ID	Primers[1]	Sequences	Tm[2] (°C)	Amplicon length[3] (bp)
LP-1	For (17657521)	5′-CAATGTAGCTAAAGCACAATTATTTCAT-3′	63.5	2180
	Rev (17659663)	5′-CACAGAATTAATCCCATATCTTATTTGA-3′		
LP-2	For (17659324)	5′-TCTATGACTCCTTCACTTAAAAGTGTTT-3′	63.5	2470
	Rev (17661775)	5′-TAGGATTTCTAAGTCAGTATGTGCATTT-3′		

[1]For: Forward primer, Rev: Reverse primer. Primer-binding sites in the chicken genome (GenBank accession No: LOC414338) are presented in parentheses. [2]The optimized annealing temperature at which a single amplicon of the expected size was obtained. [3]Length in base pairs (bp) of the expected amplicon based on the binding sites of the forward and reverse primers

amplicon was purified using Diffinity RapidTip (Diffinity Genomics, Inc., West Henrietta, NY) and sequenced (VBI, Blacksburg, VA) using the BigDye Terminator, Version 3.1, Sequencing kit (Applied Biosystems, Carlsbad, CA). The sequences were analyzed for SNPs using Phred, Phrap, Polyphred and Consed as previously described by Guan *et al.*[18].

Statistical analyses: Allele, genotype and haplotype frequencies were determined by standard counting. The computer program Arlequin ver 3.5[20] was used to estimate pairwise linkage disequilibrium (D´) among SNP loci, to test genotype frequencies for Hardy-Weinberg equilibrium (HWE) and to estimate the fixation index (F_{ST}) between the two lines. Within lines F_{ST} for each locus was estimated using the following Eq:

$$F_{ST} = \frac{H_T - H_S}{H_T}$$

where, H_T is the expected heterozygosity for line A and B birds and H_S is the expected average heterozygosity in line A or B.

Haplogroups were manually determined based on the output from Visual Haplotype (VH1) software (http://gvs.gs.washinton.edu/GVS/). The following model was used for the analysis of the relationship among gene expression, WG, LS and haplogroups:

$$Y = \mu+G+L+S+(G\times L)+(G\times S)+ e$$

where, Y is the trait measured and estimated on chicken (*chLEAP-2)* gene expression, WG and LS: (data were taken from Casterlow *et al.*[12] study), μ is the overall population mean, G is the fixed effect of genotype, L is the fixed effect of lines, S is the fixed effect of sex, (G×L) is the interaction between the genotype and lines, (G×S) is the interaction between the genotype and sex and e is the residual error. Data were analyzed using PROC GLIMMIX of SAS (SAS Inst. Inc., Cary, NC). The values were presented as least square mean±standard error. Associations were considered significant at $p\leq0.05$.

RESULTS AND DISCUSSION

The amplicons produced by the two primer-pairs spanned a 4.6 kb region that included the *chLEAP-2* gene (Table 1). A total of 16 SNPs were detected in the sequences scanned including two in the exons, 7 in the introns and 7 in the promoter region. The complete list of the SNPs, their sequence contexts, alleles and GenBank identification (*dbSNP*) numbers are presented in Table 2. While 6 SNPs have previously been reported, 10 SNPs represented novel nucleotide variants. One of the SNPs detected in the exons was non-synonymous involving an amino acid codon change from cysteine to tyrosine at 21 amino acid (aa) position of the *chLEAP-2* peptide. As expected, most of the SNPs were C-T/A-G transitions. Within the 82 birds screened, the minor alleles ranged in frequency from 0.01-0.47 with the observed heterozygosity of 0.02 and 0.50, respectively. Approximately 25% of the SNPs were in HWE ($p\geq0.05$). F_{ST} estimated for the 16 loci ranged from 0.01-0.36 and from 0.01-1.00 for lines A and B, respectively (Table 2). The pairwise F_{ST} value for the two genetic lines was 0.17 ($p=0.03$). The relatively low F_{ST} estimate suggests low genetic differentiation between the two genetic lines and probably a reflection of their common ancestry.

Across all SNPs, D´ ranged from 0.01-1.00. The correlation coefficient (r^2) for the SNPs ranged from 0.001-0.75 (Table 3).

Table 2: Characteristics of single nucleotide polymorphisms (SNPs) identified in the chLEAP-2 gene in two divergent commercial chicken lines

SNPs	Location	Nucleotide position[1]	Sequence context[2]	db identification[3]	Genotype	Genotype frequency (%)	MAF[4]	HWE[5]	F_{ST}[6] Line A	F_{ST}[6] Line B
cL2-1	Promoter	17657601	GAACC(*C*/T)GACAC	rs313602475**	C/C	20.7	0.41	NS	0.07	0.17
					C/T	42.7				
					T/T	36.6				
cL2-2	Promoter	17657628	GTTAC(*A*/T)CTGCA	rs317671313**	A/A	32.9	0.34	0.00*	0.36	0.04
					A/T	2.5				
					T/T	64.6				
cL2-3	Promoter	17657694	GGATG(*T*/C)ATAAA	rs313333274**	C/C	36.6	0.45	0.01*	0.09	0.16
					C/T	37.8				
					T/T	35.6				
cL2-4	Promoter	17657769	CACAC(*C*/T)ACTCC	rs15710013	C/C	34.2	0.36	0.00*	0.09	0.05
					C/T	3.6				
					T/T	62.2				
cL2-5	Promoter	17658065	ATGGA(*A*/G)CAACC	rs13505626**	A/A	4.9	0.05	0.00*	0.08	0.04

Table 2: Continue

SNPs	Location	Nucleotide position[1]	Sequence context[2]	db identification[3]	Genotype	Genotype frequency (%)	MAF[4]	HWE[5]	F_{ST}[6] Line A	F_{ST}[6] Line B
					A/G	0.0				
					G/G	95.1				
cL2-6	Promoter	17658137	ACTGC(*A*/G)CATGA	rs13505627	A/A	1.2	0.01	0.00*	0.01	1.00
					A/G	0.0				
					G/G	98.8				
cL2-7	Promoter	17658183	AAGTA(*A*/T)TAACG	rs14065137**	A/A	35.4	0.35	0.00*	0.26	0.09
					A/T	0.0				
					T/T	64.6				
cL2-8	Intron 1	17659251	CCTGG(*G*/T)CTGCT	ss316597681**	G/G	15.9	0.16	0.00*	0.26	0.13
					G/T	0.0				
					T/T	84.1				
cL2-9	Intron 1	17659298	GATGA(*G*/T)CATTG	rs14065141	G/G	23.2	0.38	0.00*	0.19	0.38
					G/T	26.8				
					T/T	50.0				
cL2-10	Intron 1	17659347	CTCCT(*T*/C)CACTT	rs14065142	C/C	43.9	0.38	NS	0.20	0.04
					C/T	38.8				
					T/T	18.3				
cL2-11	Intron 1	17659377	GACTC(*G*/A)AGCAT	rs14065143	A/A	39.0	0.42	NS	0.12	0.01
					A/G	36.6				
					G/G	24.4				
cL2-12	Intron 1	17659394	ATGAC(*C*/T)CATGC	rs14065144	C/C	3.5	0.04	0.01*	0.29	0.08
					C/T	2.6				
					T/T	93.9				
cL2-13[7]	Exon 2	17659452	GGTGT(*A*/G)CTGTG	rs313347050**	A/A	23.2	0.42	NS	0.07	0.14
					A/G	37.8				
					G/G	39.0				
cL2-14	Exon 2	17659533	AGACC(*C*/T)GTTGG	rs315357131**	C/C	30.5	0.43	0.00*	0.13	0.03
					C/T	25.6				
					T/T	43.9				
cL2-15	Intron 2	17659622	ATGCT(*C*/T)GATGC	rs314725270**	C/C	50.0	0.47	0.00*	0.05	0.04
					C/T	7.3				
					T/T	42.7				
cL2-16	Intron 2	17659626	TTGAT(*A*/G)CTGTG	rs313584346**	A/A	29.3	0.34	0.00*	0.16	0.04
					A/G	8.5				
					G/G	62.2				

[1]Position of the SNP in Ensembl on the forward strand of chromosome 13 of the *Gallus gallus* genome sequence. [2]Within each sequence context, alleles at the SNP locus appear in parentheses. The minor allele is italicized in the parentheses. [3]rs prefix indicates the SNP that has been previously reported in the National Centre for Biotechnology Information (NCBI) database of SNPs, rs** prefix indicates novel SNPs detected in the present study. [4]Minor allele frequency (MAF) of 16 SNPs markers. [5]Significance of deviation from HWE for the 16 SNPs. NS indicates non-significant ($p>0.05$) while * refers to significant at $p<0.05$. [6]Fixation index (F_{ST}) estimates for each of the 16 SNPs. [7]Non synonymous variant which changes the codon from TAC to TGC (cysteine to tyrosine)

Table 3: Linkage disequilibrium as measured by D´ and r^2 between the 16 segregating SNPs in the *chLEAP-2* gene

SNPs[1]	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1		0.91	0.21	0.47	NS	NS	0.45	0.56	0.29	0.84	NS	NS	0.17	0.17	0.38	0.39
2	0.29		1.00	0.35	1.00	NS	0.19	0.34	0.81	0.42	0.29	0.75	0.96	0.21	0.47	0.56
3	0.04	0.43		0.44	NS	NS	0.27	0.52	0.82	NS	0.64	NS	0.92	0.37	0.44	0.55
4	0.09	0.11	0.09		NS	NS	0.75	0.71	0.42	0.31	NS	1.00	0.48	NS	NS	NS
5	NS	0.03	NS	NS		1.00	0.61	0.39	NS	NS	0.57	NS	NS	NS	NS	1.00
6	NS	NS	NS	NS	0.24		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
7	0.08	0.04	0.03	0.54	0.04	NS		NS	0.49	0.28	NS	1.00	0.38	NS	NS	NS
8	0.04	0.05	0.05	0.06	0.04	NS	NS		0.62	0.65	0.82	NS	0.49	0.49	0.77	0.36
9	0.07	0.21	0.50	0.06	NS	NS	0.08	0.05		0.32	0.61	NS	0.77	0.32	0.45	0.34
10	0.31	0.15	NS	0.03	NS	NS	0.03	0.14	0.04		NS	1.00	NS	NS	0.46	0.32
11	NS	0.07	0.24	NS	0.02	NS	NS	0.19	0.16	NS		NS	0.72	0.61	0.81	0.36
12	NS	0.04	NS	0.06	NS	NS	0.07	NS	NS	NS	NS		1.00	NS	NS	NS
13	0.03	0.43	0.75	0.09	NS	NS	0.06	0.04	0.51	NS	0.27	0.03		0.43	0.48	0.55
14	NS	0.03	0.09	NS	NS	NS	NS	0.07	0.05	NS	0.35	NS	0.10		0.67	0.23
15	0.11	0.10	0.18	NS	NS	NS	NS	0.11	0.14	0.12	0.41	NS	0.19	0.30		0.57
16	0.07	0.25	0.16	NS	0.03	NS	NS	0.04	0.04	0.10	0.12	NS	0.14	0.04	0.18	

[1]SNP identification (*cL2-1-cL2-16*) (see Table 2). NS in the table indicates non-significant ($p\geq0.05$) D′ and r^2 values. D′ values are listed in upper right section and r^2 values are listed in lower left section

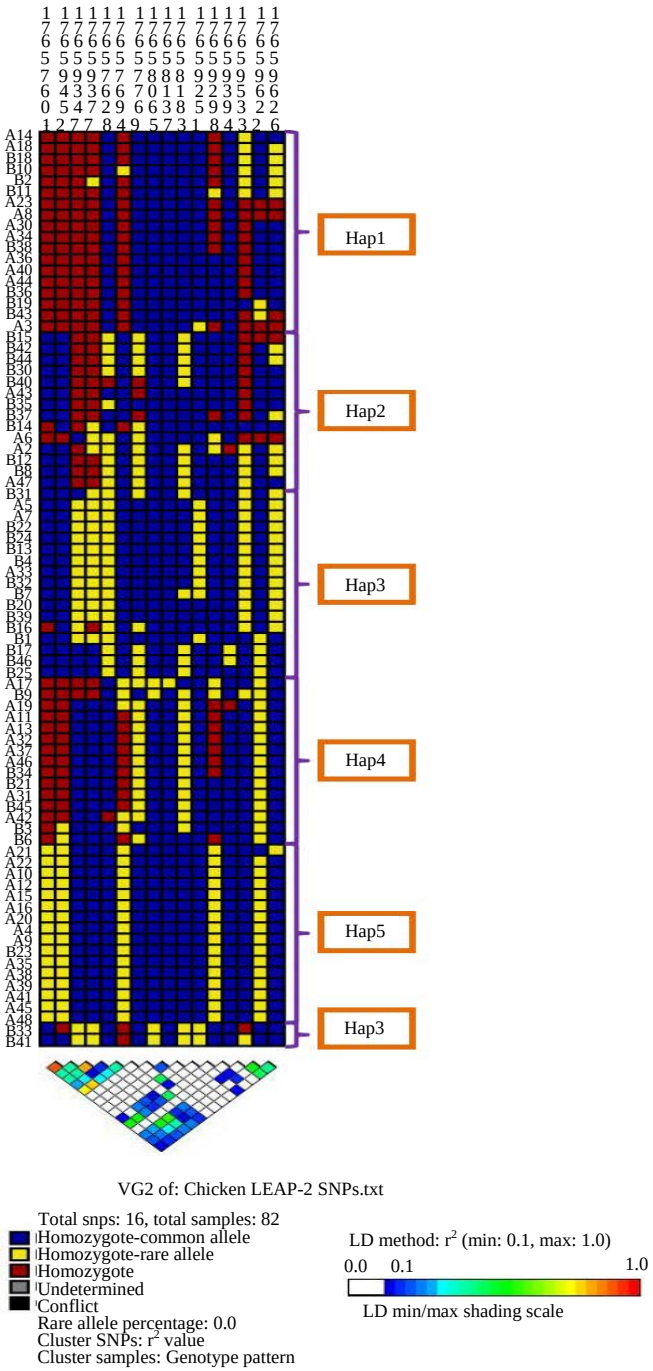

Fig. 1: Visualization of haplogroups using visual haplotype (VH1) software. (http://gvs.gs.washinton.edu/GVS/)

The haplotypes observed from the 16 SNPs were grouped into five haplogroups (Appendix: Fig. 1). The haplogroups ranged in frequency from 0.17-0.23 in the combined chicken lines (Table 4). The most common haplogroups were Hap1 and Hap3 with frequencies of 0.22 and 0.23, respectively. The frequencies of Hap1, 4 and 5 were greater in line A, while those of Hap2 and 3 were greater in line B (Table 4).

The average down-regulation of *chLEAP-2* expression of the haplogroups in lines A and B ranged from 3.89- to 27.70-fold and 9.68- to 108.29-fold, respectively (Table 4). Hap3 and Hap5 showed the greatest average down-regulation in line A (27.70-fold) and line B (108.29-fold), respectively. Hap4, on the other hand, showed the lowest average down-regulation in lines A (3.89-fold) and B (9.68-fold) respectively (Table 4).

Table 4: Haplogroups frequency, gene expression, lesion score and weight gain

Haplogroups		Frequency		Expression±SE[3**]		Lesion score±SE[4**]		Weight gain (g)±SE[5**]	
ID[1]	N[2]	Line A	Line B	Line A	Line B	Line A	Line B	Line A	Line B
Hap1	18	0.12	0.10	6.63±12.63	16.19±13.66	1.72±0.51	2.48±0.55	268.91±21.11	208.43±22.84
Hap2	14	0.04	0.13	11.83±19.85	14.89±12.85	1.71±0.81	2.17±0.52	232.25±33.17	209.60±21.48
Hap3	19	0.04	0.19	27.70±24.08	40.46±9.83	1.91±0.98	2.25±0.40	226.05±40.23	222.95±16.42
Hap4	15	0.11	0.07	3.89±13.15	9.68±15.92	0.85±0.53	1.53±0.65	276.28±21.98	230.05±26.61
Hap5	16	0.18	0.02	4.30±9.85	108.29±39.92	1.52±0.40	3.77±1.60	252.66±16.46	115.91±65.71

[1]Hap1-C-T-T*-T-G-G-T-T-G*-C-A*-T-G-C*-C*-A*-, Hap2-T-A*-C-C*-G-G-A*-T-T-T-G-T-G-C*-C*-A*-, Hap3-T-A-C-T*-G-G-T*-G*-T-T*-G*-T-G-C*-C*-A*-, Hap4-C-T-T*-C*-G*-G*-A*-T-T*-C-A-T-A*-T*-T-G-, Hap5-C-T-T-T-G-G-T-T-G-C-A-T-A-T-T*-G*-. The variant nucleotide within each haplogroup is represented by *(Alternate alleles are shown in Table 2). [2]Number of birds (N = 82) within each haplogroup. [3]Values are expressed as LS means of *chLEAP-2* gene expression±SE of birds within each haplogroup. Down-regulation of intestinal *chLEAP-2* was determined by real-time PCR using the $2^{-\Delta\Delta Ct}$ method and is indicated as negative fold change values[12]. [4]LSmeans of LS±SE of birds within each haplogroup. LS ranged from 0-4. [5]LS means of WG±SE of birds within each haplogroup. Body weight was measured at the age of 14 and 20 days to estimate the weight gain. **Gene expression, LS and WG between both lines, within each haplogroup are not significantly different ($p \geq 0.05$)

Table 5: Associations between haplogroups and the gene expression and the LS and the weight gain[1]

Haplogroups	Gene expression*	LS*	Weight gain (g)*
Hap1	11.41±9.05	2.10±0.37	238.67±15.13
Hap2	13.36±12.35	1.94±0.50	220.92±20.64
Hap3	34.08±13.46	2.08±0.55	224.50±22.49
Hap4	6.78±10.03**	1.19±0.41**	253.17±16.76**
Hap5	56.29±20.35***	2.64±0.83***	184.29±34.00***

[1]Least square mean±standard error, *$p \geq 0.05$, **Represents the least susceptible haplogroup. ***Represents the most susceptible haplogroup

Interestingly for Hap5, down-regulation of *chLEAP-2* in line A was minimal (4.3-fold), whereas in line B it was the greatest (108.29-fold). Although the down-regulation of *chLEAP-2* was greater in line B than line A, there was no difference between haplotypes. The lack of statistical significance may be due to the large variation and standard errors observed.

The average LS of lines A and B for the haplogroups ranged from 0.85-1.91 and 1.53-3.77, respectively. In line A, Hap4 had the lowest LS (0.85) and Hap3, had the highest (1.91). In line B, Hap4 had the lowest LS (1.53) while Hap5 had the highest (3.77) (Table 4). Differences among haplogroups for LS were not statistically significant ($p \geq 0.05$).

The average WG (g) of lines A and B during the challenged period for the haplogroups ranged from 226.05-276.28 and 115.91-230.05, respectively. In line A, Hap3 had the lowest WG (226.05 g) and Hap4, had the highest WG (276.28 g). In line B, Hap5 had the lowest WG (115.91 g) while Hap4 had the highest WG (230.05 g) (Table 4). Although there was a difference for WG between the lines, there was no significant difference among haplogroups for WG ($p \geq 0.05$). The average *chLEAP-2* expression of the combined chicken lines for haplogroups ranged from 6.78- to 56.29-fold. The average LS of the combined chicken lines for the haplogroups ranged from 1.19-2.64. The average WG (g) of the combined chicken lines for the haplogroups ranged from 184.29-253.17 (Table 5). These results suggest that the association among *chLEAP-2* expression levels, LS, WG and haplogroups are inconsistent.

In the current study *chLEAP-2* gene was selected as a marker for genetic resistance to coccidiosis based on the earlier study where Casterlow *et al.*[12] reported that two genetically selected chicken lines (A and B) showed differential *chLEAP-2* expression in response to an *E. maxima* challenge with line A birds exhibiting higher resistance. In addition, they showed that *chLEAP-2* gene was more down-regulated in line B compared to line A. For an immunological trait, in this study, LS was selected which is one of the best methods to assess the effect of coccidiosis in poultry[21]. The body weight gain was used as one of the economical traits where infection leads to reduced WG resulting in severe economic losses for the poultry industry[12]. In the present study, new variants were described in the *chLEAP-2* gene and used them to identify haplotypes and haplogroups that span this gene. Of the variants identified, we found one non-synonymous variant in exon 2 of the *chLEAP-2* gene which involving an amino acid codon change from cysteine to tyrosine at 21 amino acid (aa) position of the chLEAP-2 peptide. The mature chLEAP-2 peptide is encoded in exon 2 of the chicken gene as a 40 aa peptide (amino acids 37-76)[11] suggesting that the variant which we detected was not within the mature peptide encoded region of the chLEAP-2 and therefore it has no effect on the mature peptide. Haplotypes were constructed with the 16 SNPs which were used to develop the haplogroups. We analyzed the association between the haplogroups and lines for *chLEAP-2* gene expression, LS and WG. The results showed that differences among the haplogroups for gene expression, LS and WG were not significant ($p>0.05$). However, line A showed the lowest *chLEAP-2* gene expression, LS and the highest WG

compared to line B across all haplogroups. Although not significant, Hap4 appeared to be the haplogroup least susceptible to coccidiosis (lowest average down-regulation of *chLEAP-2*, lowest mean LS and highest mean WG) while Hap5 was most susceptible. These results suggest that chLEAP-2 does not play a major role in regulating coccidiosis, but may be one of a number of genes that together regulate a coccidial infection.

In this study, we only examined the role of cis-acting SNPs in *chLEAP-2* expression. However, further studies are needed to determine if trans-acting factors explain the differences previously reported in *chLEAP-2* expression in lines divergent for susceptibility to coccidiosis. Earlier work by Casterlow *et al*.[12] showed that line B birds had, on average, higher LS and a greater down-regulation of *chLEAP-2* than line A following *E. maxima* challenge. Sumners *et al*.[13] also reported that expression of *chLEAP-2* in *E. praecox* infected birds was significantly decreased in the duodenum and jejunum. Interestingly, the expression of *chLEAP-2* in the small intestine and liver increased significantly in 5 day-old birds infected with *Salmonella enterica*[11]. Michailidis[9] also reported that *chLEAP-2* is developmentally regulated during chicken embryonic development, is constitutively expressed in the chicken epididymis and is induced in the chicken gonads in response to *Salmonella enteritidis* infection.

CONCLUSION

Results of the current study do not support the hypothesis that chicken from selected lines susceptible to *E. maxima* would possess a common *chLEAP-2* haplogroup. No significant association among *chLEAP-2* expression levels, LS, WG and *chLEAP-2* haplogroups was observed in the current study. The identification of particular haplogroups that are less affected by *E. maxima* is vital for management and breeding purposes in poultry. The information presented in the current study, would be useful for future genotype: phenotype evaluation studies between *chLEAP-2* and other traits in the chicken using a candidate gene approach.

ACKNOWLEDGMENT

The authors wish to thank Virginia Polytechnic Institute and State University, USA and Department of Animal and Poultry Sciences of this institute for providing the necessary facilities for this study.

REFERENCES

1. McDougald, L.R., 2003. Diseases of Poultry. 11th Edn., Iowa State University Press, Ames, pp: 973-1023.
2. Schnitzler, B.E. and M.W. Shirley, 1999. Immunological aspects of infections with *Eimeria maxima*: A short review. Avian Pathol., 28: 537-543.
3. Cuperus, T., M. Coorens, A. van Dijk and H.P. Haagsman, 2013. Avian host defense peptides. Dev. Comp. Immunol., 41: 352-369.
4. Zhang, G. and L.T. Sunkara, 2014. Avian antimicrobial host defense peptides: from biology to therapeutic applications. Pharmaceuticals, 7: 220-247.
5. Satchell, D.P., T. Sheynis, Y. Shirafuji, S. Kolusheva, A.J. Ouellette and R. Jelinek, 2003. Interactions of mouse Paneth cell α-defensins and α-defensin precursors with membranes prosegment inhibition of peptide association with biomimetic membranes. J. Biol. Chem., 278: 13838-13846.
6. Pigeon, C., G. Ilyin, B. Courselaud, P. Leroyer, B. Turlin, P. Brissot and O. Loreal, 2001. A new mouse liver-specific gene, encoding a protein homologous to human antimicrobial peptide hepcidin, is overexpressed during iron overload. J. Biol. Chem., 276: 7811-7819.
7. Krause, A., R. Sillard, B. Kleemeier, E. Kluver and E. Maronde *et al*., 2003. Isolation and biochemical characterization of LEAP-2, a novel blood peptide expressed in the liver. Protein Sci., 12: 143-152.
8. Smith, J., D. Speed, A.S. Law, E.J. Glass and D.W. Burt, 2004. *In-silico* identification of chicken immune-related genes. Immunogenetics, 56: 122-133.
9. Michailidis, G., 2010. Expression of chicken LEAP-2 in the reproductive organs and embryos and in response to *Salmonella enterica* infection. Vet. Res. Commun., 34: 459-471.
10. Townes, C.L., G. Michailidis and J. Hall, 2009. The interaction of the antimicrobial peptide cLEAP-2 and the bacterial membrane. Biochem. Biophys. Res. Commun., 387: 500-503.
11. Townes, C.L., G. Michailidis, C.J. Nile and J. Hall, 2004. Induction of cationic chicken liver-expressed antimicrobial peptide 2 in response to *Salmonella enterica* infection. Infect. Immunol., 72: 6987-6993.
12. Casterlow, S., H. Li, E.R. Gilbert, R.A. Dalloul, A.P. McElroy, D.A. Emmerson and E.A. Wong, 2011. An antimicrobial peptide is downregulated in the small intestine of *Eimeria maxima*-infected chickens. Poult. Sci., 90: 1212-1219.
13. Sumners, L.H., K.B. Miska, M.C. Jenkins, R.H. Fetterer, C.M. Cox, S. Kim and R.A. Dalloul, 2011. Expression of Toll-like receptors and antimicrobial peptides during *Eimeria praecox* infection in chickens. Exp. Parasitol., 127: 714-718.

14. Su, S., K.B. Miska, R.H. Fetterer, M.C. Jenkins and E.A. Wong, 2014. Expression of digestive enzymes and nutrient transporters in *Eimeria acervulina*-challenged layers and broilers. Poult. Sci., 93: 1217-1226.
15. Su, S., K.B. Miska, R.H. Fetterer, M.C. Jenkins and E.A. Wong, 2015. Expression of digestive enzymes and nutrient transporters in *Eimeria*-challenged broilers. Exp. Parasitol., 150: 13-21.
16. Yin, H., L.H. Sumners, R.A. Dalloul, K.B. Miska and R.H. Fetterer *et al*., 2015. Changes in expression of an antimicrobial peptide, digestive enzymes and nutrient transporters in the intestine of *E. praecox*-infected chickens. Poult. Sci., 94: 1521-1526.
17. Gilbert, E.R., H. Li, D.A. Emmerson, K.E. Webb Jr. and E.A. Wong, 2007. Developmental regulation of nutrient transporter and enzyme mrna abundance in the small intestine of broilers. Poult. Sci., 86: 1739-1753.
18. Guan, X., T. Geng, P. Silva and E.J. Smith, 2007. Mitochondrial DNA sequence and haplotype variation analysis in the chicken (*Gallus gallus*). J. Heredity, 98: 723-726.
19. Rozen, S. and H. Skaletsky, 2000. Primer3 on the WWW for General Users and for Biologist Programmers. In: Bioinformatics Methods and Protocols (Methods in Molecular Biology, Volume 132), Krawetz, S. and S. Misener (Eds.). Humana Press, Totowa, NJ., USA., ISBN-13: 9780896037328, pp: 365-386.
20. Excoffier, L. and H.E.L. Lischer, 2010. Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and windows. Mol. Ecol. Resour., 10: 564-567.
21. Johnson, J. and W.M. Reid, 1970. Anticoccidial drugs: Lesion scoring techniques in battery and floor-pen experiments with chickens. Exp. Parasitol., 28: 30-36.