STUDIES ON MHC HAPLOTYPING IN GUINEA FOWL AND ITS ASSOCIATION WITH GENERAL IMMUNOCOMPETENCE

THESIS

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CERTIFICATE

Certified that **DR. ATUL GUPTA** a candidate of **M.V.Sc. (final)** examination of **2008** in ***"Animal Genetics and Breeding"*** has been working under my supervision during this session and that the accompanying thesis entitled, **"STUDIES ON MHC HAPLOTYPING IN GUINEA FOWL AND ITS ASSOCIATION WITH GENERAL IMMUNO-COMPETENCE"** which he is submitting, is his genuine work.

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Date: 01/09/2008

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Atul Gupta

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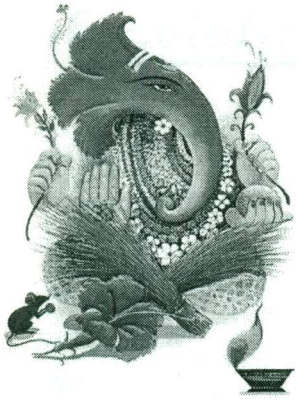
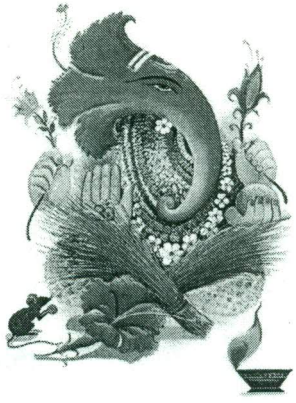


Dedicated to...

My Beloved Parents

&

Brothers



Abbreviations

°C	Degree Celsius
µg	Microgram
µl	Microlitre
µM	Micromolar
bp	Base pair
cm	Centimeter
CMI	Cell mediated immunity
D	Day
A.D.W.	Autocalved Distilled water
A	Adenine
G	Guanine
C	Cytosine
T	Thymine
dATP	Deoxy adenosine triphosphate
dCTP	Deoxy cytosine triphosphate
dGTP	Deoxy guanosine triphosphate
dTTP	Deoxy thymidine triphosphate
DNA	Deoxyribose nucleic acid
dNTP	Deoxy nucleoside triphosphate
EDTA	Ethylene diamine tetra acetic acid
Fig	Figure
g	Gram
HA	Haemagglutination
HI	Humoral immunity
HNO_3	Nitric acid
IC	Immunocompetence
IgG	Immunoglobulin-G
IgM	Immunoglobulin-M
i.m.	Intra muscular
Kb	Kilobase
M	Molar
MAS	Marker Assisted Selection
mg	Milligram
MHC	Major histocompatibility complex
Min	Minute

ml	Millilitre
mm	Millimeter
mM	Millimolar
MW	Molecular weight
N	Number
NDV	Newcastle disease virus
ng	Nanogram
nm	Nanometer
OD	Optical density
p.i.	Post immunization
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PCR-RFLP	PCR–restriction fragment length polymorphism
pH	Hydrogen ion concentration
RE	Restriction enzyme
rpm	Rotations per minute
SDS	Sodium dodecyl sulphate
Sec	Seconds
SRBC	Sheep red blood cells (sheep erythrocytes)
PCR-SSCP	PCR-Single strand conformation polymorphism
TBE	Tris borate EDTA
TE	Tris – EDTA
U	Units
u.v.	Ultraviolet
V	Volts

Contents

Introduction

Introduction

Guinea fowl (*Numida meleagris*), an important poultry species comes under family Numidae and order galliformes. Guinea fowl differs from the fowl not only in their phenotypic appearance but also in behavioral and production characteristics. One very important characteristic of guinea fowl is its resistance to the common diseases occurring in chicken (Aitken *et al.*, 1977), which make guinea fowl an important model for studying the mechanism of disease resistance.

The immune system is the natural means by which individual resist infection, and immunological parameters may reflect the immuno-competence of the immune system and, in turn, the ability of the individual to resist infection. So far, only a few genes have been identified in influencing disease resistance. Genes from the Major Histocompatibility Complex (MHC) involved in antigen-presentation were among the earliest set of genes to be identified. These genes encode some of the most polymorphic bio-molecules known in the animal kingdom. The MHC (Major Histocompatibility Complex) in chickens, initially known as 'B Complex' (Briles *et al.*, 1950a) is an extensively polymorphic erythrocyte antigen or blood group system. The chicken major histocompatibility complex (MHC) is known to have a very strong association to disease resistance and susceptibility to numerous

pathogens including Marek's disease virus (Hansen et al., 1967; Briles et al., 1977), Rous sarcoma tumor virus (Bacon et al., 1981), avian leukosis virus (Yoo and Sheldon 1992), Pasteurella multocida (Lamont et al., 1987), coccidia (Lillehoj et al., 1989), and salmonella (Cotter et al., 1998). Regulation of cellular communication in the immune response is a critical function of chicken MHC (Kaufman and Lamont, 1996).

The MHC has widespread effects on genetic control of immune responsiveness either due to its role as restriction element or through specific MHC linked immune response. Antibody titers to several soluble antigens (e.g. Salmonella pullorum and sheep erythrocytes) are also associated with chicken MHC. Hence, MHC has been used as a set of candidate genes for association between DNA markers and antibody response (Yonash *et al.*, 2000). Understanding MHC and its association with disease resistance and immune responsiveness can allow genetic selection to be performed on the basis of MAS rather than by direct challenge of population with disease agent.

The chicken MHC is commonly identified with polyclonal antisera produced by immunizations between birds having different haplotypes (Briles and Briles 1982; Juul-Madsen et al., 2006). The MHC serological reactivity is due to both BG antigens and BF-encoded class I molecules. BG and BF genes are in linkage disequilibrium with each other and with BL (MHC class II genes). The MHC haplotype nomenclature was standardized initially using serologic reagents (Briles et al., 1982). A recent nomenclature update ties standard haplotypes with gene sequences and experimental flocks (Miller et al., 2004). These haplotypes are identified in inbred lines, with the majority being derived from the White Leghorn (WL) breed. Serological typing is fraught with cross-reactivity (Fulton et al., 1995) that can complicate application of the technique to out bred populations (Kroemer et al., 1990). The presence

of cross-reactive MHC epitopes, additional polymorphic non-MHC antigens, and the existence of novel haplotypes in these contribute to this problem. Inbred lines contain a limited combination of BG, BF, and BL genes. In out bred populations, novel alleles and combinations of alleles are likely to exist. This additional level of variation results in production difficulties for haplotype-specific antisera which can yield inaccurate haplotype identification.

With the advent of molecular biology tools, B haplotypes can now be determined with several methods including two-dimensional (2-D) gels (Miller et al., 1984), restriction fragment length polymorphism (RFLP) (Miller et al., 1988; Juul-Madsen et al., 1993; Emara et al., 2002; Landesman et al.,1993; Iglesias et al., 2003), DNA sequence (Sung et al., 1993; Miller et al., 2004), single-strand conformation polymorphism (Goto et al., 2002), and sequence-specific polymerase chain reaction (SS-PCR) (Zheng et al., 1999; Livant and Ewald 2005). These tools have also been applied to study MHC diversity in broiler-type chickens (Li et al., 1997, 1999; Livant et al., 2001; Livant and Ewald 2005). These methods identify either protein (2-D gels) or DNA differences within very defined regions (RFLP, amplified fragment length polymorphism, and SS-PCR) producing very consistent and repeatable results among labs. Unfortunately, these techniques are not always practical for large numbers of samples.

Very recently, Fulton et al. (2006) used the microsatellite marker LEI0258 which is known to be physically located within the MHC, between the BG and BF regions. DNA from various serologically defined MHC haplotypes was amplified by polymerase chain reaction with primers surrounding this marker. Twenty-six distinctive allele sizes were identified. Some serologically well-defined MHC haplotypes shared a common LEI0258 allele size but could be distinguished either by the

addition of information from another nearby marker (MCW0371) or by small indels or single nucleotide polymorphism (SNP) differences between the alleles. They found that the association between LEI0258 allele and serologically defined MHC haplotype was very consistent for the same haplotype from multiple sources. Sequence information for the region defined by LEI0258 was obtained for 51 different haplotypes. Two internal repeats whose lengths were 13 and 12 bp, respectively, are the primary basis for allelic variability. Allele size variation ranges from 182 to 552 bp. Four indels and five SNPs in the surrounding sequence provide additional means for distinguishing alleles. Typing with LEI0258 and MCW0371 was useful in identifying MHC haplotypes in out bred populations of chickens particularly for the initial development of serological reagents.

There is no report on MHC haplotyping in Guinea fowl. Hence, an attempt has been made to generate preliminary data on MHC haplotyping using the procedure described by Fulton et al. (2006) and to find any association with immunocompetence as well as economic traits.

Keeping the above in view, the present study was proposed with the following objectives.

1. **To generates preliminary information on MHC haplotyping in Guinea fowl through PCR based method.**
2. **To find association between MHC haplotyping and economic traits, if any.**

Review of Literature

Review of Literature

2.1 Major Histocompatibility complex

The Major Histocompatability Complex (MHC) is a set of molecules displayed on cells surfaces that are responsible for lymphocytes recognition and "Antigen presentation". The MHC molecules control the immune response through recognition of "self" and "non-self" and consequently, serve as targets in transplantation rejection. In human, the MHC is encoded by several genes located on chromosome 6. Class I molecules are encoded by the BCA region while class II molecules are encoded by D region. A region between these two on chromosome 6 encodes class III molecules, including some complement components.

2.2 Avian Major Histocompatibility complex

Briles *et al.* (1950) first described the chicken MHC as a blood group locus. The MHC *B* complex in chickens was first identified by the ability of leukocytes to give strong graft rejection (Schierman and Nordskog, 1961). Chicken was the second species in which MHC was identified (Schierman and Nordskog, 1961). From an evolutionary point of view, the most striking characteristic of MHC is the high polymorphism of some MHC loci; these loci are the most polymorphic known in animal kingdom. Chicken has 39 chromosomes, most of which are small elements called micro-chromosomes. In chicken, the MHC is located on a medium sized micro-chromosome that ranks 16th in size (Goto *et al.*, 1988).

The core region i.e. the proper MHC (B) is only 92 kb long and contains 19 genes making the chicken MHC 20 fold smaller than human MHC this region is remarkably compact, containing essential MHC genes found in mammalian counterparts (Kauffman *et al.*, 1999). They are organized differently with the class III regions genes located outside the class II and class I regions. The central portion, stretching from the class II (B-L) β genes to the class I (B-F) α genes, is especially compact with no repetitive elements or repeats. These eleven genes in roughly 44 kb have average intron sizes of 200 nucleotides, resulting in genes that are as little as one- third the size of their mammalian homologues. The important reason why chicken MHC so compact: The highly polymorphic class I and class II regions and a huge class III region does not separate genes responsible for graft rejection. Indeed it seems likely that the order of the regions in the chicken MHC is primordial indicating that mammalian MHC arose by rearrangement (Kaufman *et al.*, 1999).

A great deal of work has been done to sequence and clone portions of the chicken MHC. This resulted in the confirmation of three chromosomal regions or loci: BF, BL and BG, which make up the B complex (Bourlet *et al.*, 1988; Goto *et al.*, 1988; Guillemot *et al.*, 1988), producing class I, II and IV antigens respectively. The three loci of the B complex are tightly linked. No cases of recombination between the B-F and B-L loci have been found (Guillemot *et al.*, 1988), but several cases of recombination are known to have occurred between the B-G and B-F/B-L portions (Hala *et al.*, 1981).

2.3. Identification of MHC haplotypes

2.3.1. Traditional methods

Typing for B haplotypes has traditionally been accomplished by serogical methods, which identify gene products, expressed on

erythrocytes (haemagglutination). Serological typing for MHC haplotypes has several advantages among which are high throughout and detection of expressed genes. However, disadvantage of using alloantisera is that reagents are complex mixtures of antibodies and are non-renewable and require characterization, adsorption and validation of each new lot. Moreover, in chickens' alloantisera that are specific for given B haplotypes within a line often exhibit extensive cross-reaction when used in other lines of chickens (Simonsen *et al.,* 1989)

Fulton et al. (1996) used chicken MHC alloantiserum cross-reactivity analysis by haemagglutination and flow cytometry for identifying MHC haplotypes. They reported the analysis of 53 alloantisera made with MHC congenic lines. They observed that erythrocytes specific cross reactive antigens attribute to B-G molecules and were demonstrated for B5:B19, B12:B19 and B19:B21 and B19:B21 cross-reactions.

2.3.2. Molecular approaches

Modern molecular biological tools have replaced traditional methods of typing or identifying MHC alleles or haplotypes. These technologies enable researchers to conduct analysis of the molecular structure of genes and gene products of MHC. One of these new technologies is the analysis of DNA by restriction fragment length polymorphism on Southern blots (Lamont *et al.,* 1987) or by other methods.

Anderson *et al.* (1987) investigated Class II genes of MHC in the chicken by Southern blot analysis using human cDNA probes for DQ α, DQ β, DR α and DR β. They observed excellent correlation between these RFLP types and the serological B typing since RFLP type was identical within each pair of homozygote.

Hala *et al.* (1988) attempted to detect recombination between B-F and B-L genes within the chicken B complex by serological typing, *in vitro* MLR and RFLP analysis. The absence of detectable recombination was confirmed by restriction fragment length polymorphism analysis with B-L β and B-F probes. They arrived at decision that distance between B-F and B-L loci is below 0.01 centi Morgan.

Miller *et al.* (1988) used chicken B-G sub region cDNA assigned probes to analyze restriction fragment length polymorphisms of the chicken MHC. In every instance genotyping by RFLP pattern was found to confirm the B-G allele assigned serologically. The technique was also used by Piteovski *et al.* (1989) to analyze B-G region and immune response genes in chicken using hybridization of sperm deoxyribonucleic acid with a major histocompatibility complex Class II probe.

Hala *et al.* (1989) used RFLP for typing MHC haplotypes in Obese Strain (OS) chicken. They analyzed the genetic background of autoimmune thyroiditis using OS chicken model. They observed that haplotypes B5 and B15 were associated both with severe as well as mild infiltration.

Lamont *et al.* (1990) analyzed the restriction fragment length polymorphism of the major histocompatibility complex of 1515-B congenic chicken lines. They suggested that description of MHC Class I and II restriction patterns of well-characterized 1515B congenic lines will aid in identification of genes important in diseases resistance. RFLP analysis in addition to serological B typing may sharpen the tools in the search for recombinant chromosomes separating B-F and B-L.

Juul *et al.* (1992) have analyzed seven serologically defined restriction fragment length (RFLP) with chicken cDNA probes specific for MHC Class I and II. They demonstrated an excellent correlation

between the observed RFLP banding pattern in the investigated haplotypes and serological B typing.

Plachy *et al.* (1992) opined that molecular genotyping of recombinant congenic lines provides evidence for crossing over within the B-G region of major histocompatibility complex.

Miller and Goto (1993) used single-stranded conformational polymorphism ("PCR-SSCP") assay to study the expression of B-G genes in non-erythroid tissues.

Goto *et al.* (2002) have developed a DNA-based method for defining MHC *B* system genotypes in chickens. Genotyping by this method requires neither prior determination of allele-specific differences in nucleotide sequence nor the preparation of haplotype-specific alloantisera. Allelic differences at chicken *B-F* (class I) and *B-L* (class II) loci are detected in PCR single-strand conformation polymorphism (SSCP) assays. PCR primer pairs were designed to hybridize specifically with conserved sequences surrounding hypervariable regions within the two class I and two class II loci of the *B*-complex and used to generate DNA fragments that are heat- and formamidedenatured and then analyzed on nondenaturing polyacrylamide gels. PCR primer pairs were tested for the capacity to produce SSCP patterns allowing the seven *B* haplotypes in the MHC *B* congenic lines, and seven *B* haplotypes known to be segregating in two commercial broiler breeder lines to be distinguished. Primer pairs were further evaluated for their capacity to reveal the segregation of *B* haplotypes in a fully pedigreed family and in a closed population. Concordance was found between SSCP patterns and previously assigned MHC types. *B-F* and *B-L* SSCP patterns segregated in linkage as expected for these closely linked loci. We conclude that this method is valuable for defining MHC *B* haplotypes and for detecting potential recombinant haplotypes especially when used in combination with *B-G* (class IV) typing by restriction fragment pattern.

2.4. PCR-RFLP studies for MHC genes

Warner *et.al.* (1989) conducted RFLP analysis of MHC class II genes from inbred lines by using restriction enzymes(*Pvu* II, *Hind* III, *Bgl* II and *Bam* HI).They found that birds with the same haplotype always showed the same RFLP pattern ;however some birds with the different haplotypes always showed the same RFLP pattern.

Uni *et al.* (1995) compared the frequencies of MHC-RFLP markers and haplotypes with early immune traits in the divergent lines of chicken selected for high and low antibody response to *E.coli* vaccinated at 10 days of age. Their results showed implied a relationship between class IV genes and early antibody production.

Juul-Madsen *et al.* (1997) determined new chicken Rfp-Y haplotypes by the use of restriction fragment length polymorphism (RFLP) and mixed lymphocyte culture (MLC) in four different chicken haplotypes, B15, B19, B21 and B201. For the first time it is shown that major histocompatibility complex class II genes in the Rfp-Y system have functional implications.

Lamont and Weigend (1999) selected chickens of four lines divergently for high (H) and low (L) immunocompetence in replicate and analyzed them to investigate polymorphisms of MHC class II and MHC class IV on the molecular level associated with selection. The *Sac* I digested DNA was hybridized individually with MHC class II and MHC class IV gene probes. The MHC class II RFLP analysis revealed four polymorphic bands The hybridization with the MHC class IV probe displayed 26 scorable bands, of which 18 were polymorphic.

Ouyang-jianhua *et al.* (2000) studied the genetic variation of the MHC in Chinese taihe silky fowl by PCR-RFLP using 4 restriction enzymes (*Hha* I, *Eco*RV, *Hae* III, and *Xba* I). The MHC genotype classified on the

basis of combination of all the restriction enzyme had non-significant relationship with egg weight and hatching rate of fertile eggs, and the polymorphism detected by *Eco*RV and *Xba* I were not related to egg production and fertility rate.

Ahmed (2001) developed high and low titer lines against response to sheep red blood cell i.e. HSRBC and LSRBC, respectively and high and low responder lines to Cell mediated immune-response against PHA-P i.e. HCMI and LCMI lines, respectively in a synthetic broiler dam line. He studied the polymorphism in the β_1 exon of the BLBII gene between HSRBC and LSRBC; and between HCMI and LCMI lines. He adopted the specific primers used by Zheng *et al.* (1999) to amplify a 235 bp fragment. However, using *Taq* I and *Hae* III restriction enzymes, he could not get the polymorphism between these lines.

Shivakumar (2003) studied the polymorphism in the $\hat{a}_1$ exon of the BL B II gene in the divergent lines for response to sheep red blood cell in white leghorn chicken. He adopted the specific primers used by Livant *et al.* (2001) to amplify a 267 bp fragment. However, using *Taq* I restriction enzyme, he could not get the polymorphism between high titer line and low titer line in IWG as well as in IWJ lines of White Leghorn.

Muthukumar (2003) studied the 2nd generation of HSRBC, LSRBC, HCMI and LCMI lines in broiler. Using the primers adopted by Ahmed (2001), he amplified a 235 bp fragment, representing $\hat{a}_1$ exon of the BL BII gene. He studied polymorphism using PCR-RFLP between these lines, however using *Taq* I, he also could not get polymorphism between these lines.

Ahmed (2007) studied the polymorphism in the $\hat{a}_1$ exon of the BL BII gene of turkey and polymerase chain reaction–restriction fragment-length polymorphism analysis of exon 2 using the *Hinf* I restriction

enzyme demonstrated three restriction patterns and a preliminary evidence of multiple β loci in turkey. PCR-RFLP analysis of turkey MHC class II loci could be a promising method of MHC genotyping. Turkey MHC haplotypes identified earlier by RFLP analysis should be sequenced to standardize turkey MHC nomenclature and to develop DNA based method of haplotyping.

2.5. General Immunocompetence Status

The immune system triggers several processes that protect the host against the non-self when an antigenic substance is introduced within the body. The immunological defense mechanism may be categorized into three different but interdependent functional facets (i) the humoral system, which gives life to humoral immunity or antibody production, (ii) the cellular system, that stimulates cell mediated immunity or direct cellular intervention against pathogens and (iii) the non specific systems comprising of complement system phagocytic cells and lysozyme activity. The primary cellular constituents of immune systems are bursa derived (B) and thymus derived (T) lymphocytes and phagocytic cells (macrophage and granulocytes). It is the interaction between macrophages, T-cells and B-cells that provide the optimum immune responses to chickens with a complete spectrum of resistance processes.

The genetic control of disease resistance is complex and involves several systems of the body with the immune system being an important component (Warner *et al.*, 1987). Selection for resistance to particular disease tends to be specific and has little effect on general disease resistance required in modern poultry production (Gavora, 1990). Correlated responses to selection for antibody titres to foreign non-pathogenic proteins provides resistance to a wider range of diseases

than would be achieved by selection for individual disease resistance (Boa-Amponsem *et al.* 1997). Biozzi *et al.* (1979) concluded that all the three facets of immune systems are under the control of separate quantitative trait loci. Results of Van der Zijpp (1983) from experiments in chickens were in agreement with this suggestion. Therefore, considering several immunological parameters simultaneously could make improvement for genetic resistance to disease development.

2.5.1. Antibody Response to Sheep Red Blood Cells (SRBC)

Efforts were made by several workers to identify a common genetic marker that can be used for selection of birds for general immune competence (Gavora and Spencer, 1983; Van der Zijpp, 1983). One such system that aroused considerable interests in poultry is the selection based on antibody response to SRBC.

The immune response to natural, non-pathogenic, non-specific, multi-determinant complex antigens like sheep red blood cells may provide good indication of natural immune status of an individual.

The response to sheep red blood cells is T -cell dependent and has association with B-G region of major histocompatability and with one dominant gene of gene complex. The antibody response against SRBC has been widely used to measure the general immunocompetence (Siegel and Gross, 1980; Vander Zijpp and Leenstra, 1980; Vander Zijpp *et al.*, 1983; Ubosi *et al.*, 1985a, b; Kundu *et al.*, 1999; Haunshi, 1999).

Among all the immunoglobulin isotypes (viz. IgG, IgM, IgE, IgD, IgA), the IgG is the major portion of an antibody response and different from IgM in its ability to neutralize viral infectivity, bacterial infectivity, interactions with other immune components and serological reactions including haemagglutination (Barret, 1983). The application of 2-Mercapto-ethanol produces a comparative estimate of antibody activity

and not of actual quantities of 7 S and 19 S antibodies produced (OsIer, 1978). The assumption that the destruction of the -SH bonds only affects the reactivity of IgM antibodies still remains controversial. Despite these limitations, the 2-ME treatment of serum to estimate the 2-ME resistant and susceptible antibody titre as a measure of IgG and IgM antibody titre, respectively has been used by various workers such as Vander Zijpp and Leenstra (1980), Vander Zijpp *et al.* (1983), Ubosi *et al* (1985a and b), Martin et al. (1989), Kundu (1997) and Haunshi (1999)

In an experiment, Van der Zijpp and Leenstra (1980) measured total HA, 2 MER (IgG) and 2 MES (IgM) antibody titre against SRBC in seven weeks old WLH chickens on day 0, 3, 7 and 13-post injection of SRBC intramuscularly. The effects of sex and hatch were significant on mean titre of sheep RBC. Females showed a significantly higher response than males. The mean titre was 5.2 (Log 2) on day 7-post injection.

Zijpp *et al.* (1986) compared Cornish, White Leghorn, North Holland blue and Friesian fowl strains for humoral, cell mediated and phagocytic immune response. There was significant strain effect for humoral response to SRBC and phytohaemagglutinin-stimulated swelling of wing web. Boa-Amponsem *et al.*, (1991) observed that males from a heavier meat line had lower antibody response to SRBC than those of lighter line.

Dunnington *et al.* (1987) evaluated the early (K^+) and late feathering chicks of broiler lines for antibody titre against SRBC at 35 days of age. They could not find any significant difference between early feathering and late feathering genotypes and also effect of sex or diet was non-significant on antibody response to SRBC.

Petrovsky *et al.* (1988) revealed genetic differences in humoral response to *Br. abortus* and SRBC in hens of four commercial lines of

White Leghorn, RIR, White Leghorn breeds and twelve groups if their crosses immunizing simultaneously at 14 months of age. In the WL breed the responsiveness of *Br. abortus* was higher, whereas to SRBC it was lower. A closer affinity to the immunological reactions of the paternal line was observed in the hybrids with both the antigens.

Miller *et al.* (1992) reported that additive genetic variation of both primary and secondary immune response in chicken. Reciprocal differences and heterosis was also observed to influence the secondary response. Saxena (1993) compared guinea fowl, Kadaknath chicken and broiler for antibody response to sheep red blood cells. The overall mean anti-SRBC titre [$\log_{10}(n+1)$] in guinea fowl, Kadaknath and broilers were 1.520±0.487, 1.525±0.068 and 1.386±0.12 respectively. Significant differences were also observed among varieties and sire families for response to SRBC. However, the effects of sex and interaction were non-significant.

Boa-Amponsem *et al.* (1997) studied the genetic architecture of antibody response to sheep red blood cells in chicken by producing parental, reciprocal F_1, F_2 and backcross progenies from two lines of WLH selected divergently for high (HH) and low (LL) antibody response on 5th day post injection of 0.1 ml of 25% SRBC. They observed that female progenies of the HHLL F_1-cross exhibited the mean heterosis of 22%. Although maternal effects generally had no influence on antibody titres, maternal heterosis in the selected trait was observed due to sex linkage.

Kundu (1997) studied response to SRBC in different poultry populations such as Aseel, Kadaknath, Naked neck, Frizzle, Dahlem Red, White Leghorn, Synthetic Dam line of broilers and naked neck broilers on 0, 5, 12, 19 days post SRBC immunization. He observed that

all genetic groups showed highest titre on 5 dpi, except broilers, which had peak titre at 12 dpi. Dahlem Red showed highest titres for total SRBC and 2-MER responses.

Nath (1999) evaluated four synthetic lines of broiler namely colored synthetic male line (CSML), white synthetic male (CSFL) line, colored synthetic female (CSFL) line and naked neck (NNL) line and their crosses for total antibody, 2 MER and 2 MES response to SRBC. Significant differences were observed among all genetic groups for total SRBC, MER and MES response. However, the effects of sex and interaction effects of sex and genetic groups were non-significant on the total SRBC response.

Haunshi and Sharma (2002) measured the antibody response to SRBC in four pure chicken breeds viz. Dahlem Red (DR), Aseel (AS), Kadaknath (KN) and White leg horn (WLH) and their selective crosses such as DR x WLH, DR x AS and DR x KN at 10-12 weeks of age. Significant differences were observed among pure breeds; Dahlem Red showed higher total HA titre (11.30±0.39) and 2MER titre (7.3±0.45). However, crossbreds did not show any significant differences for total HA antibody titre.

Haunshi *et al.* (2002) studied the effect of naked neck (Na) and Frizzle gene (F) on antibody response to SRBC in BC_2 populations of chicken at 10-12 weeks of age. The naked neck and frizzle genes did not seem to influence the antibody response to SRBC.

2.6. Association of MHC with immune competence

A critical function of the chicken major histocompatibility complex (MHC) is the regulation of cellular communication in the immune response. Pevzner *et al.* (1979) observed antibody production against a variety of antigens was linked to chicken MHC. Antibody titers to several soluble antigens (e.g. bovine serum albumin, BSA), to viral antigens

and to cellular antigens (e.g. *Salmonella pullorum* and sheep erythrocytes) are also associated with the chicken MHC. Total serum IgG levels are also under genetic control by the B complex.

The cell surface proteins of MHC, which serve to distinguish self from non-self, are unique to each genetically unique individual. Vainio *et al.* (1984) used cyclophosphamide (CP) model to study T-B cell interaction in the chicken to determine the genetic requirements. Administration of CP in the neonatal period causes a permanent depletion of B cell compartment.

The use of MHC recombinant chicken lines demonstrated that the Class II (B-L) antigens alone were the necessary restriction elements in T-B cell cooperation for antibody production (Vainio *et al.,* 1984). Qureshi *et al.* (1986) showed that the chemotactic activity of chicken blood mononuclear leucocytes and the activity and recruitment to the peritoneal cavity of macrophages varied among B congenic lines which differ from each other only in the microchromosome (or a fraction of it) which bears the MHC. Germain (1986) reported that MHC Class II genes encode proteins that present antigens to helper cells and thus are critical in initiating the cell mediated immunity against invading pathogens.

The MHC has profound effects on genetic control of immunoresponsiveness via its role as a restriction element or via specific immune response genes. The studies have been supported by the fact that a molecule can stimulate immune response only if it binds to groove of the MHC a phenomenon known as "MHC restriction" (Vainio *et al.,* 1987). Thus the phenomenon is very important for some of different cell types of the immune system. To communicate effectively they must share at least one haplotype. Since Class I and II histocompatibility molecules receive antigenic epitopes via two different processing pathways so their

antigen presentation activities are directed to two different classes of T cells *i.e.* killer and helper T cells. This division has an important significance as regards infections like the activities of cytotoxic CD8+, T cells can thereby be directed towards any Class I bearing cells in body. CD4+ helper, T cells on the other hand react with epitopes borne by Class II positive cells such as macrophages and B cells which themselves require signals from T cell to become activated to produce antibody. Dietert *et al.* (1987) emphasized that the control of MHC over communication events can occur at two levels; molecular interactions at the cell surface and cellular cooperation in an immune response.

The MHC has been studied in chickens line selected for various traits of immunosuppressiveness for example in experiments to measure long term selection for antibody response to sheep red blood cells. Correlated changes of MHC allelic frequencies with the antibody selection occurred. Dunnington *et al.* (1989) reported that although MHC genotype explains part of variation in antibody levels, the background genome also had a substantial effect.

Heller *et al.* (1991) provided serological evidence for major histocompatibility B complex in broiler selected for humoral immune response. Genetic selection for early humoral immune responsiveness against two antigens *i.e.* heat killed *E.coli* and Newcastle disease virus vaccine was performed in a heterogenic population of broiler chicken. Chicken lines congenic for MHC were also used to demonstrate MHC associations with CD4 and CD8 lymphocyte percentage and ratios (Hala *et al.,* 1991). They reported the possibility of a direct association between the genetic characteristic represented in the B complex and humoral antibody response in a broiler population.

Crittenden (1991) emphasized the importance of virus-derived genes for inducing virus resistance. They illustrated this by resistance of transgenic chickens expressing the avian leukosis virus envelope genes

to superinfection by the same virus. Dunnington *et al.* (1992) studied the antibody responses to combinations of antigens in White Leghorn chickens of different background genomes and major histocompatibility complex genotypes. Their studies reveal that antibodies to NDV were lower in chickens of MHC genotype B21B21.

Parmentier *et al.* (1996) reported divergent responses to intramuscularly administered *E.coli*, NDV, IB, IBD and divergent body weight of chicken lines selected for high and low humoral responsiveness to SRBC (sheep red blood cells). They suggested that selection for SRBC resulted in enhanced responsiveness to components of several vaccines. Cahaner *et al.,* (1997) have recently used various probes and shown that Tap2 and B-F regions were associated with antibody production.

Macrophages are essential cells for initiating antibody production in addition to being phagocytic. The influence of chicken major histocompatibility complex (MHC) on adherence potential of monocyte derived macrophages was examined using the congenic chicken lines by Hala *et al.*(1998). These lines represent well-defined genetic models for the study of resistance or susceptibility to the progressive growth of Rous Sarcomas. It was concluded that the gene-regulating adherence potential is localized within B-F/L region of chicken MHC.

Yonash *et al.* (2000) examined MHC as a set of candidate genes associated between DNA markers and antibody response. They analyzed the association between RFLP bands and three antibody response traits (*E.coli*, Sheep RBC and Newcastle diseases virus). The MHC class IV probe was highest in polymorphism but had lowest number of bands associated with antibody traits. They further observed TAP2 probe yielded20different RFLP bands of which five were associated with multiple antibody response traits. However, MHC class I probe yielded 15

polymorphic bands of which five were associated with antibody production. Thus, their study illustrated the efficacy of using multiple MHC regions probes as candidate markers for quantitative trait loci controlling antibody response in chicken.

Materials and Methods

Materials and Methods

3.1. Resource population & measurement of Immunocompetence Traits

A closed flock of Lavender variety of guinea fowl maintained at Experimental Guinea Fowl farm at CARI, Izatnagar was used. 100 guinea fowl birds (straight run) were utilized for experimental work. Apart from guinea fowl, 15 individuals from each of the three chicken breeds i.e. White Leghorn (WL), Aseel (AS) and Red Cornish (RC) were also used.

The general innate immuno-competence status of in 100 guinea fowl birds was assayed by measuring antibody response to SRBC at the age of 10 weeks. The microtitre plate haemagglutination procedure as described by Siegel and Gross (1980) with slight modifications was followed to measure total HA antibody titres in these birds on day zero as well as on 9th day post injection. The procedure followed was as follows-

3.1.1. Preparation of Sheep Red Blood Cells (SRBC) Suspension

Blood from jugular vein of healthy sheep was collected in Alsever's solution. The red blood cells were washed thrice in PBS (phosphate buffer saline, pH 7.2). Finally 1% suspension of SRBC in PBS (V/V) was prepared.

3.1.2. Immunization and Harvesting of Immune Serum

1 ml of 1% (V/V) of SRBC suspension was injected intravenously to each bird. About 3 ml of blood on 0 and 9th day post-immunization were collected from jugular vein. The blood was allowed to clot in an incubator at 37°C for 1 hour. The clot was allowed to retract after detaching it from sides of its container & left at 4°C. Centrifugation of blood was carried out at 2000 rpm for 5-10 minutes to facilitate rapid collection of the serum. Required quantity of immune serum was harvested and stored at -35°C.

3.1.3. Haemagglutination Test (HA Test)

Haemagglutination (HA) test was used to determine total HA antibody titre. The microtitre plates (U bottom) were cleaned, rinsed with PBS and dried. The HA test was done in duplicate for each sample. In each well of microtitre plate, 50 µl of PBS was distributed. Then, 50µl of serum was added in the first well. Two fold serial dilutions were made up to row 11. The 12th row was kept as control. 50 µl of a 1% SRBC suspension in PBS was added in each well. The plates were covered and shaken gently on automatic shaking machine to enhance proper mixing. The microtitre plates were then kept at 37°C for 1 hour for incubation. The plates were read under bright light. The titre was expressed as $\log_2$ of the highest dilution in which there was complete haemagglutination. The response titre were the result of the difference between HA titre before and after SRBC immunization.

3.2. Extraction of Genomic DNA

About 0.5 ml of blood was collected from Jugular vein in 1.5ml-eppendorf tube containing EDTA from each individual of the guinea fowl as well as three chicken breeds i.e. WL, AS & RC. All the blood samples were stored at –20 °C till further processing. The high molecular

weight genomic DNA was isolated using the following simple method. To 50 µl of blood, 700 µl of lysis buffer (10 mM Tris. HCl, 100 mM NaCl, 1 mM EDTA, pH: 8.0 and 0.5% SDS) containing 60 µg of proteinase K (20 mg/ml) was added. The mixture was vigorously vertexed and incubated at 37°C for 10-12 hours with gentle shaking. The DNA was purified by extracting with equal volume of phenol, phenol-chloroform and chloroform-isoamylalcohol (24:1). The genomic DNA was precipitated by adding 0.1 volume of 3 M sodium acetate and two volumes of ice chilled ethanol and centrifuged for 5 minutes at 14000 g. DNA pellet was then washed with 70% ethanol, air-dried and subsequently resuspended in 200 µl TE buffer (10 mM Tris. HCl, 1mM EDTA).

3.2.1. Purity, Concentration and Quality of Genomic DNA

After the complete dissolution of DNA, its optical density at 260 and 280 nm was determined by UV spectrophotometry. The purity of DNA was checked by taking the ratio of optical density at 260 and 280 nm. The DNA samples having O.D ratio between 1.7 and 1.9 have been used for the study.

It is known the one O.D. unit at 260 nm equals 50 µg/ml of pure, double stranded DNA. Therefore, the concentration of DNA samples was calculated by the following formula

Concentration (µg/ml) = O.D. at 260 nm x Dilution ratio x 50

The quality of genomic DNA was examined by horizontal electrophoresis of DNA samples on 0.8 % agarose gel. Loading samples were prepared by adding approx. 1.0 - 2.0 µg of DNA, 2 µl of 6X Bromophenol blue and 5 µl of distilled water. Electrophoresis was performed at 2 V/cm (Max. 5 V/cm of gel) for 1 to 2 hrs. Finally the gel was examined under UV light. The good quality DNA samples having intact DNA bands without any smearing were selected for further analysis.

3.3. Microsatellite Assay

3.3.1. Selection of Marker

The primer LEI 0258 was used for MHC haplotyping as suggested by Fulton et al (2006). The sequence of the forward and reverse primer is presented in table 3.1. These primers were synthesized commercially.

Table 3.1. Sequence of the forward and reverse primer used to amplify the Lei 0258 locus

Sl No	Type of primer	Sequence (5'-3')
1	Forward	TCG GGA AAA GAT CTG AGT CAT TG
2	Reverse	TGA TTT TCA GAT CGC GTT CCT C

3.3.2. PCR Amplification

Initially the amplification conditions, reported in literature for this marker was tested. Subsequently, the PCR amplification conditions were standardized. Amplification reactions were carried out in a final volume of 25 µl reaction mixtures in 0.2 ml thin wall PCR tubes. Each PCR tube containing 25-50 ng genomic DNA, 1.5 mM Mgcl2. 50 mM Kcl, 10 mM Tris-Hcl, pH=8.8, 0.1% Triton X-100, 0.01% gelatin, 200 µM of each dNTP (dATP, dGTP, dCTP and dTTP), 1.0unit of Taq DNA polymerase enzyme and 10 pico mole of each forward and reverse primer.

The amplification was carried out in a thermocycler (Eppendorf-Germany). Protocol for PCR reaction consisted of an initial denaturation at 94°C for 5 min. Followed by 35 cycles of PCR, each cycle consisting of 45 s at 92°C, 45 s at 57 °C and 45 sec at 72°C, and followed by a final extension step of 1.5-h at 72 °C. Molecular sizes of various alleles of were estimated by using 100 bp DNA ladder (Bangalore Genei).

3.3.3. Resolution and documentation of Microsatellite alleles

The amplification products from the microsatellite markers were resolved on 3.5 % metaphor agarose gel. The gels were stained with ethidium bromide and photographed under UV light.

3.4. SSCP Assay

3.4.1. Amplification of 277 bp fragment

A set of primer was designed to amplify the β1 domain of BLB2 gene using the chicken BLB2 sequence (Table 3.2).

Table 3.2. Details of the primers used for amplifying the 277 bp fragment

Primer Set	Primers	Primer Sequence (5' - 3')	Expected fragment size bp
Set I	Ex2-F	GTGCCCGCAGCGTTCTTC	277
	Ex2-R	TCCTCTGCACCGTGAAGG	

The amplification mixture for each sample made up to final volume of 25 µl, containing 10 pmol each primer. The further reaction was done as mentioned above. The amplification was carried out in an i-cycler (Biorad) PCR machine. The protocol for PCR reaction consisted of an initial denaturation at 94°C for 5 min, followed by 35 cycles of PCR, each cycle consisting of 45 s at 94°C, 45 s at 58°C and 45 s at 72°C. After completion, the samples were stored at 4C till further use. Molecular sizes of amplified products will be estimated by using appropriate molecular size markers.

The PCR was monitored by agarose gel electrophoresis. A 1.6 % agarose electrophoresis gel in tris-borate EDTA ("TBE") 1X buffer (89 mM Tris-borate), 89 mM boric acid (Mallinckrodt) and 2 mM EDTA was

prepared. Five to ten microliters of PCR products were loaded onto the gel with 1 µl dye 10X (0.5% bromophenol blue (Sigma), 0.5% Xylene cyanol, 40% (w/v) sucrose (IBI) in water). Electrophoresis was run in TBE 1X at 120 V for 45 minutes. The gel was stained with ethidium bromide for 5 minutes. PCR products were visualized with ultraviolet light.

3.4.2. Resolution on 10% native polyacryalmide gel

A 10 % polyacrylamide gel in TBE (0.5X) was prepared by combining (for a 6 ml gel) 3.7 ml water, 300 µl TBE 10X, 2 ml acrylamide (11.4 g acryl amide plus 0.6 g bis-acrylamide in 40 ml water), 3 µl TEMED (N,N,N',N'-tetramethylethylenediamine) and 30 µl ammonium per sulfate 10%.

PCR products (1 to 3 µl) were denatured for 5 minutes at 80° C. with 10 µl dye 1X (300 µl formamide (Fisher) plus 3 µl dye 10X). The thoroughly washed glass plates were cleaned with acetone and then with ethanol. The plates were dried thoroughly. The plates along with the spacer were fixed in the gel. Casting frame was kept in upright position. The freshly prepared gel solution (as mentioned above) was gently poured between the glass plates and was filled up to the mark noted in the notched plate. The comb was gently inserted between the plates, avoiding the trapping of air bubble between the gel and comb teeth. The gel was left for about 30 min for polymerization. The comb was removed gently. The wells were washed with 0.5 x TBE using a syringe. The plates were cleaned and removed from the gel-casting frame. The lower tank of the vertical gel electrophoresis assembly was filled with 0.5 x TBE up to the marked level. The gel plates were placed in the vertical gel electrophoresis assembly in such a way that their notched plates should face each other. The gels were fixed by placing the side

support and tightening the screws gently but firmly. The buffer was filled in the compartment made by the two plates up to the extent that the buffer should be above the notched part of both the plates. About 8 to 10 µl of the sample with dye was loaded in the well by using the gel-loading tip. The samples were loaded as such (without denaturation) in the 10% native polyacrylamide gel. Electrophoresis was run in TBE 0.5X at 200 V for 1 hour 45 minutes. After running, the gels were removed from the assembly.

3.4.3. Visualization by silver staining of the gels

The gels were placed on a clean surface by keeping their notched plate facing upwards. The upper plate was detached gently, by keeping all the care for avoiding breakage of the gel. Generally the gel remains attached with the lower plate. In case if it remains attached with the upper plate, the upper plate will be placed on a clean surface, keeping the gel on upper side. The first row loaded was marked by cutting the upper portion of the gel from that side. The gel along with the plate was placed in a suitable sized glass tray. About 500 ml of 10% ethanol was added in the tray and tray was shaken for 5 min gently to provide uniform and thorough washing of gel. Generally the gel gets detached from the plate, which was removed carefully from the tray, leaving the gel in the tray. The 10% ethanol will be removed by vacuum sucking

About 500 ml of 1% nitric acid was poured. The tray will be shaken for 3 min. The gel was quickly washed (for 20 sec.) with ADW. The 0.006 M silver nitrate solution was added to the tray. The tray was shaken for 20 min. The gel was rinsed with distilled water for few seconds. Chilled 0.028 M sodium carbonate solution with 0.038% formalin (added at the last minute) was poured in the tray and the gel was gently shaken for few minutes, until the solution becomes brownish. This step was repeated

until the bands become brownish and distinct. The reaction was stopped by adding 10% glacial acetic acid for 2 min. The gel was stored in distilled water until vacuum drying.

3.5. PCR-RFLP Assay

3.5.1. Amplification of 277 bp fragment

The 277 bp fragment was amplified as described above in 3.4.1 section.

3.5.2. RE analysis

3.5.2.1. Identification of Restriction Endonuclease Sites

The restriction enzyme map was developed for BLB2 (Exon- 2) nucleotide sequence from guinea fowl and chicken by using GENETOOL software. The map was compared and two restriction enzymes i.e. Hae III and BseG I enzymes were tabulated.

3.5.2.2. RE digestion

- About 1 µg of amplified purified fragment was digested with different restriction enzymes as per the recommendations of the manufacturer in 40 µl digestion mixture.
- About 15 µl of the digested product was resolved on 6% native polyacrylamide gel and the resolved bands were visualized through silver staining.

3.5.2.3. Resolution on 6% native polyacryalmide gel

- Preparation of 100 ml of 30% acrylamide solution

Acryalmide	29 g
Bisacrylamide	1 g

Dissolve in 100 ml of ADW.

Store at 4°C.

- Preparation of gel solution (30 ml) for 1 mm thick 13 x 13 cm sized gel.

30% acryl amide solution	6 ml
20 x TBE	1.5 ml
TEMED	80 µl
10% APS (freshly prepared)	150 µl
Auto DW	22.27 ml

- The thoroughly washed glass plates were cleaned with acetone and then with ethanol. The plates were dried thoroughly.
- The plates along with the spacer were fixed in the gel. Casting frame was kept in upright position.
- The freshly prepared gel solution (as mentioned in step 1) was gently poured between the glass plates and was filled upto the mark noted in the notched plate.
- The comb was gently inserted between the plates, avoiding the trapping of air bubble between the gel and comb teeth.
- The gel was left for about 30 min for polymerization.
- The comb was removed gently.
- The wells were washed with 1 x TBE using a syringe.
- The plates were cleaned and removed from the gel-casting frame.
- The lower tank of the vertical gel electrophoresis assembly was filled with 1 x TBE up to the marked level.
- The gel plates were placed in the vertical gel electrophoresis assembly in such a way that their notched plates should face each other.

- The gels were fixed by placing the side support and tightening the screws gently but firmly. The buffer was filled in the compartment made by the two plates up to the extent that the buffer should be above the notched part of both the plates.
- About 10 µl of the sample with dye was loaded in the well by using the gel-loading tip. The samples were loaded as such (without denaturation) in the 6% native polyacrylamide gel.The gels were run at a constant current of 11 Amp for 10-12 h.
- After running, the gels were removed from the assembly.

3.5.2.4. Visualization by silver staining of the gels

The gels stained with silver staining as described above and visualized and photographed under white light.

3.6. Statistical analysis

The mean estimates for HA titre against SRBC in three titre groups as well as in overall populations were computed and were tested for significant differences among them using standard statistical procedures (Snedecor and Cohram, 1989).

Results

Results

4.1. Development of resource population and assessment of general immune competence in guinea fowl

A resource population of guinea fowl (Lavender variety) was developed by mating of 4 males with 16 dams (4 dams with one male) and a total of 112 keets were hatched in two hatches. Out of these 112 keets, 100 randomly selected keets were used.

A total of 100 Lavender guinea fowl birds (straight run) were utilized for experimental work. The general immuno-competence status of these 100 birds was assayed by measuring antibody response to SRBC by performing HA test at the age of 10 weeks. On the basis of HA antibody titre values, these birds were divided in to three groups viz. low HA antibody titre (LHA), medium HA antibody titre (MHA) and high HA antibody (HHA) titre groups. The mean HA antibody titre values in different groups are presented in Table 4.1. The LHA birds were having a titre value ranging from 2 to 4; MHA birds were having a titre value ranging from 5 to 7, whereas HHA birds were having a titre value ranging from 8 to 10. The mean HA antibody titre was 3.36 ± 0.11 in LHA group, 5.85 ± 0.11 in MHA group and 8.53 ± 0.19 in HHA group. These estimated values differ significantly ($P>0.01$) between the groups. The overall mean was found to 5.74 ± 0.09.

4.2. DNA isolation

The high molecular weight genomic DNA was isolated from the 50 µl of whole blood collected from brachial vein in each of the 100 guinea fowl, 15 white leghorn, 15 aseel and 15 red Cornish birds using phenol-chlorophorm method (Sambrook and Russell *et al.*, 2001). The purity of DNA was evaluated by electrophoresing the samples on 0.8 % agrose gel using 1X TBE as electrophoresis buffer. The purity and concentration of genomic DNA was also checked by UV spectrophotometer taking optical density (O.D.) at 260 and 280 nm.

High molecular DNA was isolated in a good yield from all the samples of guinea fowl as well as from chicken breeds. The concentration of genomic DNA was estimated to be about 600 to 1800 ng/µl. No apparent differences were observed in DNA yield among the species/ breeds. The intact single band of high molecular weight genomic DNA and lack of any smear and contamination for all the samples reflected the high quality intact DNA. Further, the 260/280 nm absorbance ratio were from 1.7 to 1.9 for most of the samples, indicating high quality intact DNA. The DNA samples showing absorbance ratio at 260 and 280 nm between 1.7 to 1.9 were taken for further analysis. These samples were diluted to the concentration of 30 – 50 ng/µl for PCR amplification.

4.3. Microsatellite Analysis

4.3.1. Allelic profile at LEI 0258 locus in Guinea fowl and chicken breeds.

The allelic profile at LEI 258 locus in GF & three chicken breeds have been shown in Fig 4.1. In guinea fowl, monomorphic pattern was observed and only 247 allele was found for LEI 258 locus; however in chicken breeds, sizable polymorphism was observed. The numbers of alleles at this locus were 5 in White leghorn, 11 in Aseel and 7 in Red

Cornish. Allelic size range was from 205 bp to 608 bp in White leghorn, 234 bp to 590 bp in Aseel and 193 bp to 295 bp in Red Cornish.

The frequencies of different LEI0258 alleles in guinea fowl and chicken populations have been presented in Table 4.1. Among chicken breeds 608 bp allele was found specific for WL with a frequency of 0.20. The 193 bp allele with a frequency of 0.107, 220 bp allele with a frequency of 0.25 & 274 bp allele with a frequency of 0.178 were specific to RC. The numbers of specific alleles were 5 in AS i.e. 321 bp, 405 bp, 420 bp, 443 bp & 552 bp and all these alleles showed a low allelic frequencies ranging from 0.033 to 0.066. The 247 bp allele which was fixed in GF also found in AS though at a very low frequency of 0.166.

4.3.2. Probable B-haplotypes in Guinea fowl and chicken breeds

The possible B haplotypes based on LEI0258 allele size, as described by Fulton *et al.* (2006) have been presented in Table 4.2, while the predicted B haplotypes based on LEI0258 allele sizes in our guinea fowl and chicken populations have been shown in Table 4.3. In GF, only one haplotype i.e. B-18 was observed, while in chicken breeds several B-haplotypes were observed. In WL, the possible B-haplotypes were 13, 13.2, 15.2, 17, 22, 73 and BW11, while in AS Possible B-haplotypes were 1, 1.2, 2, 5, 6, 8, 11.1, 15, 15.2, 18, 19.1, 22, 29, 62, 73 & 74. In RC, the possible B- haplotypes were 2, 5, 11, 11.1, 13, 13.2, 15, 15.1, 17, 27, 29, 61 & BW11. Among all observed B-haplotyes in chicken breeds, haplotypes 1, 1.2, 19.1, 62 & 74 were found only in AS, whereas haplotype 15.1, 27 & 61 were found only in RC. None of the haplotype was found which was present only in WL.

4.4. SSCP Analysis

4.4.1. Amplification of 277 bp fragment

To amplify the β1 of BLB2 gene from genomic DNA, the primers used by Zoorab *et al.* (1993) were adopted. The forward primer was from

the intronic sequence upstream the exon 2, while the reverse primer was from the end of the exon 2 and was expected to amplify a 277 bp fragment (Fig 4.2). These chicken specific primers had successfully amplified the 277bp fragment in GF (Fig 4.3).

4.4.2. SSCP pattern in resource population

The PCR products from all the 76 GF birds were resolved on 10 % native polyacrylamide gel & the gels were stained with ethidium bromide and photographed under white light. The SSCP patterns of these individuals have been shown in Fig 4.4. A total of 6 SSCP patterns were observed depending upon the mobility of secondary structures formed by the single strands in non denaturing gel. Among all three groups in GF, the SSCP patterns from 19 individuals in low antibody titre group, 35 in medium antibody titre group & 14 individuals in high antibody titre group were used for further analysis. The SSCP patterns from 8 individuals (2 from LHA, 5 from MHA & 1 from HHA) were not clear, hence were not included in further analysis.

Numbers of individuals showing different SSCP pattern in three titre groups have been shown in Table 4.4. Among the three groups of guinea fowl population s (based on antibody titre) pattern I was shown by two individuals [29 and 40] in low antibody titre group, while in medium antibody titre group this pattern was shown by three individuals [24, 25 & 27]. In High antibody titre group the pattern I was shown by none of the individual. Pattern II was shown by two individuals [35 and 50] in medium antibody titre group, while in high antibody titre group this pattern was shown by four individuals [2, 3, 41 & 42]. In low antibody titre group the pattern II was shown by none of the individual. Pattern III was shown by five individuals [14, 15, 74, 75 and 76] in low antibody titre group and by twelve individuals [17, 19, 43, 49, 53, 54, 55, 58, 62,

66, 69 & 71] in medium antibody titre group. In High antibody titre group the pattern III was shown by four individuals [12, 70, 72 & 73]. Pattern IV was shown by only one individual [31] in low antibody titre group, while in medium antibody titre group this pattern was shown by seven individuals [48, 56, 57, 59, 60, 64 & 65]. In High antibody titre group the pattern IV was shown by five individuals [4, 9, 10, 18 & 23]. Pattern V was shown by seven individuals [7, 8, 20, 21, 22, 28 & 30] in low antibody titre group and by eight individuals [26, 32, 33, 34, 36, 37, 39 & 67] in medium antibody titre group. In High antibody titre group the pattern V was shown by none of the individual. Pattern VI was shown by four individuals [5, 6, 13 & 16] in low antibody titre group, while in medium antibody titre group this pattern was shown by three individuals [44, 47 & 52]. In High antibody titre group the pattern VI was shown by only one individual [11].

4.4.3. Proportion of various SSCP patterns in different groups

The frequencies of different SSCP patterns in three titre groups as well as overall have been presented in Table 4.5. In LHA group, pattern V was predominately present with a frequency of 0.368 followed by pattern III (0.263) & pattern VI (0.211). Pattern I and pattern IV showed comparatively lower frequencies (0.105 & 0.053 respectively), while pattern II was complete absent. In MHA group, all the 6 patterns were observed with the predominance of pattern III with a frequency of 0.343. The pattern V & pattern IV were also present in moderate frequencies i.e. 0.228 & 0.20 respectively, while the pattern I, II & VI were present in low frequencies (0.057 – 0.086).

In HHA group, only four patterns were observed as pattern I & V were completely absent. Among the remaining four patterns except pattern VI which was present in low frequency i.e. 0.073, the other

patterns were present in more or less similar frequencies ranging from 0.286 – 0.357.

4.5. PCR-RFLP pattern in resource population

4.5.1. Amplification of 277 bp fragment & RE digestion

The 277 bp fragment was amplified as described above and was digested with two restriction enzymes i.e. *Hae* III and *Taq* I as per manufacturer's instructions. The RE profile of different titre groups with these RE enzymes have been shown in Fig 4.6.

4.5.2. PCR-RFLP profile with *Hae III* restriction enzymes

The restriction enzyme profile of 30 individuals in total i.e. 10 individuals each from all three groups viz. low, medium & high antibody titre group, with *Hae III* restriction enzyme has been shown in Fig 4.6. In present study, two alleles i.e. Allele A (having RE site at 142 nt) and allele B (not having RE site) and two genotypes i.e. genotype AA (homozygous for presence of RE site) and AB (heterozygous for presence of RE site) were observed. Genotype AA was expected to show two bands i.e. one of 142 bp and another of 135 bp, but due to very small size difference between these fragments, only one thick band was shown by AA genotype. Accordingly, AB genotype was expected to show three bands i.e. one band of 277 bp and another two of 142 bp and 135 bp, but on gel only two bands were observed i.e. one of 277 bp and another thick band of ~ 140 bp. However, both the genotypes i.e. AA and AB were very easily identifiable by the presence of one band and two bands, respectively on gel. The genotype AA was shown by 4 individuals and genotype AB was shown by 6 individuals in low titre group, whereas genotype AA was shown by 6 individuals and genotype AB was shown by 4 individuals in medium titre group and genotype AA was shown by 5 individuals & genotype AB was shown by 5 individuals in high titre group. These

frequencies have been shown in Table 4.6. The frequency of these two i.e. AA & AB genotypes in different antibody titre groups in guinea fowl was 0.4 and 0.6 in low titre group, 0.6 and 0.4 in medium titre group and in 0.5 & 0.5 in high titre group respectively.

4.5.3. PCR-RFLP profile with *Taq I* restriction enzymes

The restriction enzyme profile of 30 individuals with *Taq I* restriction enzyme has been shown in Fig 4.6. In present study, two alleles i.e. Allele A (having RE site at 116 nt) and allele B (not having RE site) and two genotypes i.e. genotype AA (homozygous for presence of RE site) and AB (heterozygous for presence of RE site) were found. Genotype AA showed two bands i.e. one of 161 bp and another of 116 bp, while AB genotype showed three bands i.e. one band of 277 bp, one of 161 bp and one band of 116 bp. The genotype AA was shown by 6 individuals and genotype AB was shown by 4 individuals in low titre group, whereas genotype AA was shown by 5 individuals and genotype AB was shown by 5 individuals in medium titre group and genotype AA was shown by 5 individuals & genotype AB was shown by 5 individuals in high titre group. These frequencies have been shown in Table 4.6. The frequency of these two i.e. AA & AB genotypes in different antibody titre groups in guinea fowl was 0.6 and 0.4 in low titre group, 0.5 and 0.5 in medium titre group and in 0.5 & 0.5 in high titre group respectively.

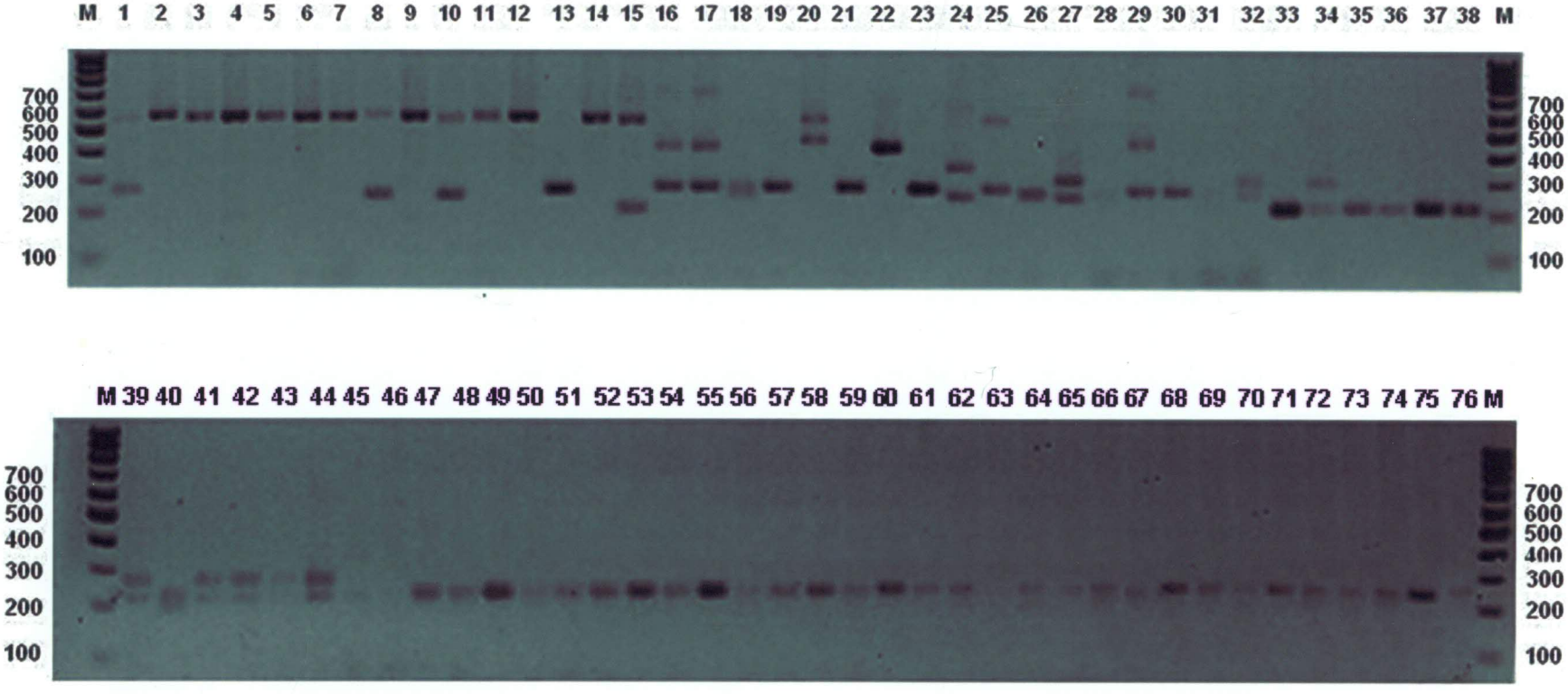


Fig. 4.1 . Allelic profile at LEI0258 locus in White Leghorn (lane 1-15), Aseel (Lane 16-30), Red Cornish (Lane 31-45) and Guinea fowl (lane 46-76). M : Molecular size marker (100 bp ladder)

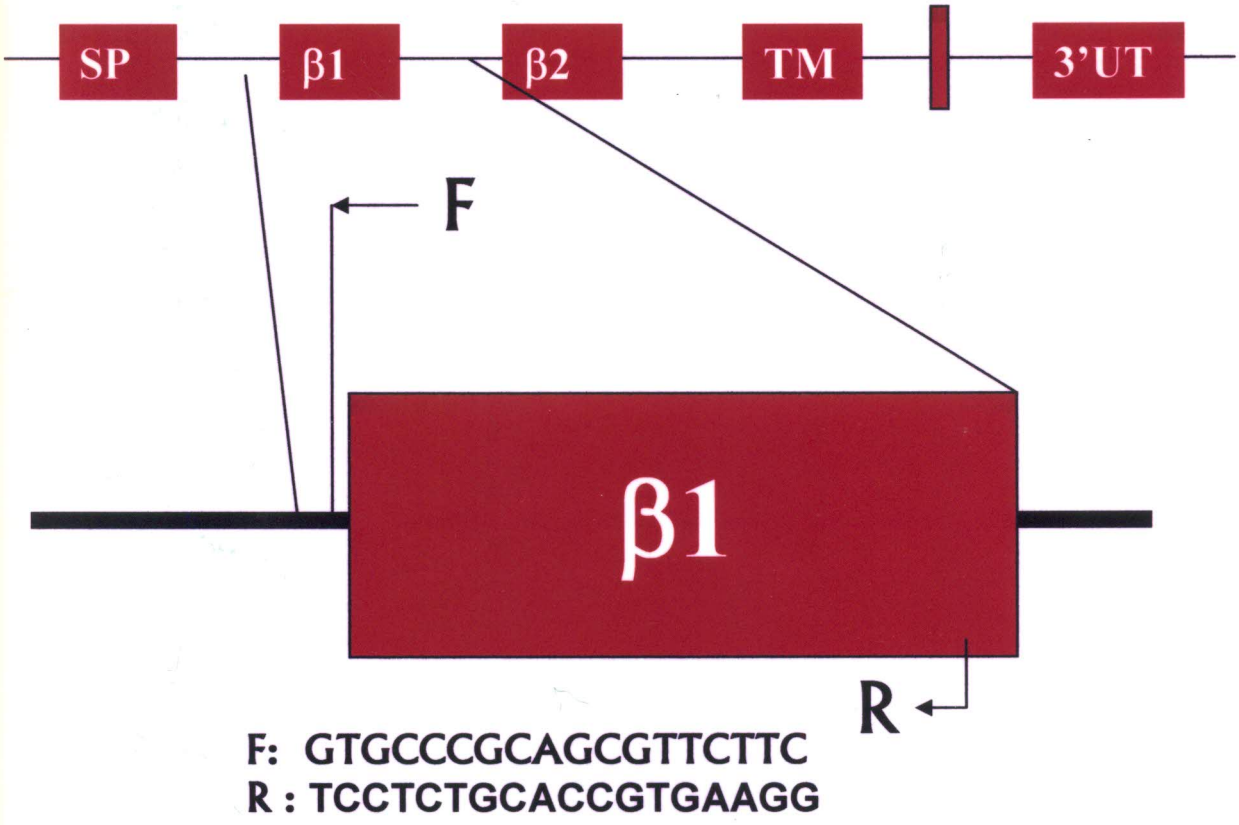


Fig 4.2. Organization of BLB2 gene and primers used for amplification of β1 exon of BLB2 gene

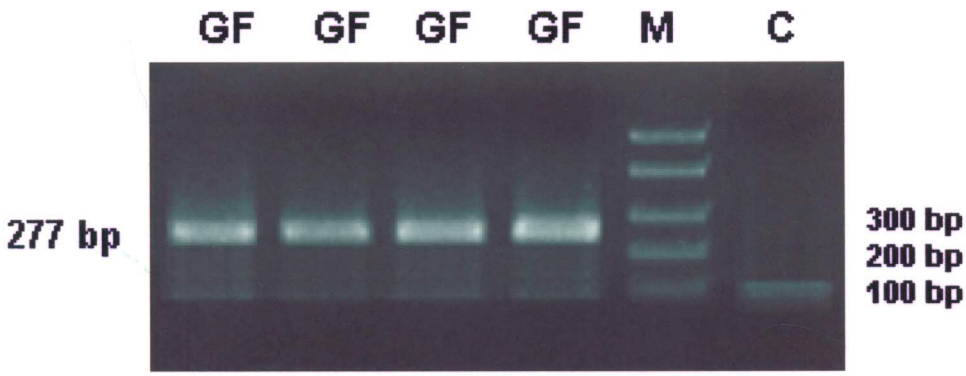


Fig 4.3. Amplification of 277 bp in Guinea fowl (GF : Guinea Fowl; C : Negative control M : Fast ruler low range, Fermentas)

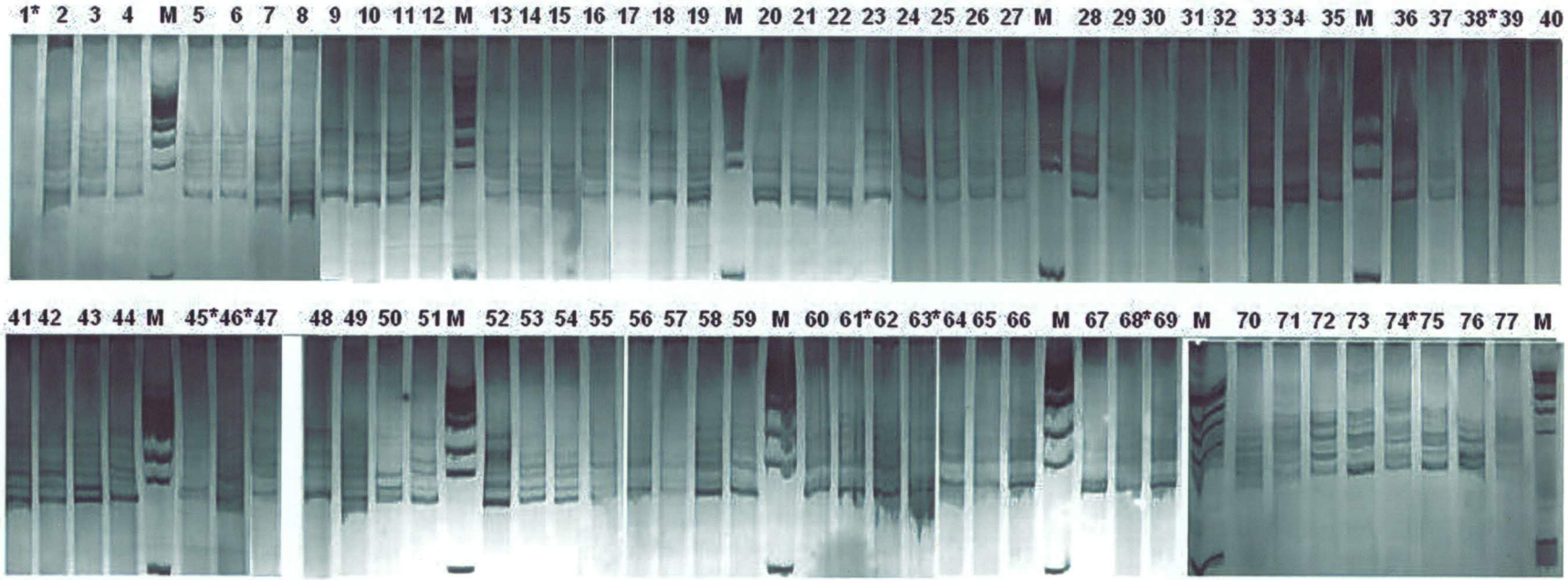
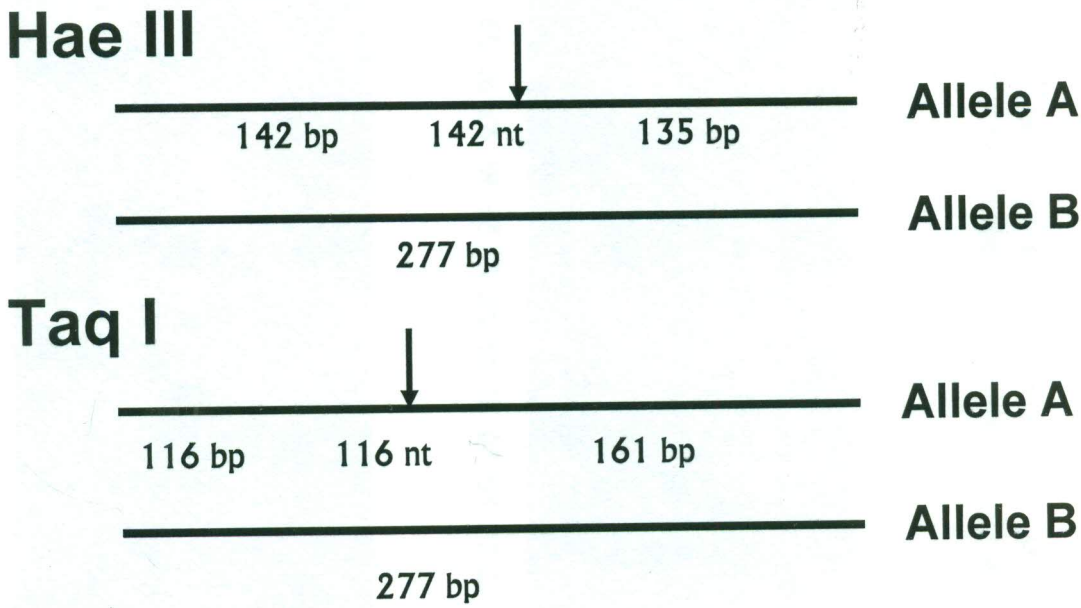


Fig. 4.4. SSCP patterns in various HA titre group in Guinea fowl. (LHA: 5, 6, 7, 8, 13, 14, 15, 16, 20, 21, 22, 28, 29, 30, 31, 40, 51, 75, 76; MHA: 17, 19, 25, 26, 27, 32, 33, 34, 35, 36, 37, 39, 43,44, 47, 48, 49, 50, 52, 53, 54, 55, 56, 57, 58, 59, 60, 62, 64, 65, 66, 67, 69, 71; HHA: 2, 3, 11, 12, 14, 41, 42, 70, 72, 73)

Note - * patterns are not clear



Selected REs	Expected sizes (bp)	
Hae III	142	135
Taq I	161	116

Fig. 4.5 Digramatic representation of different alleles with different restriction enzymes

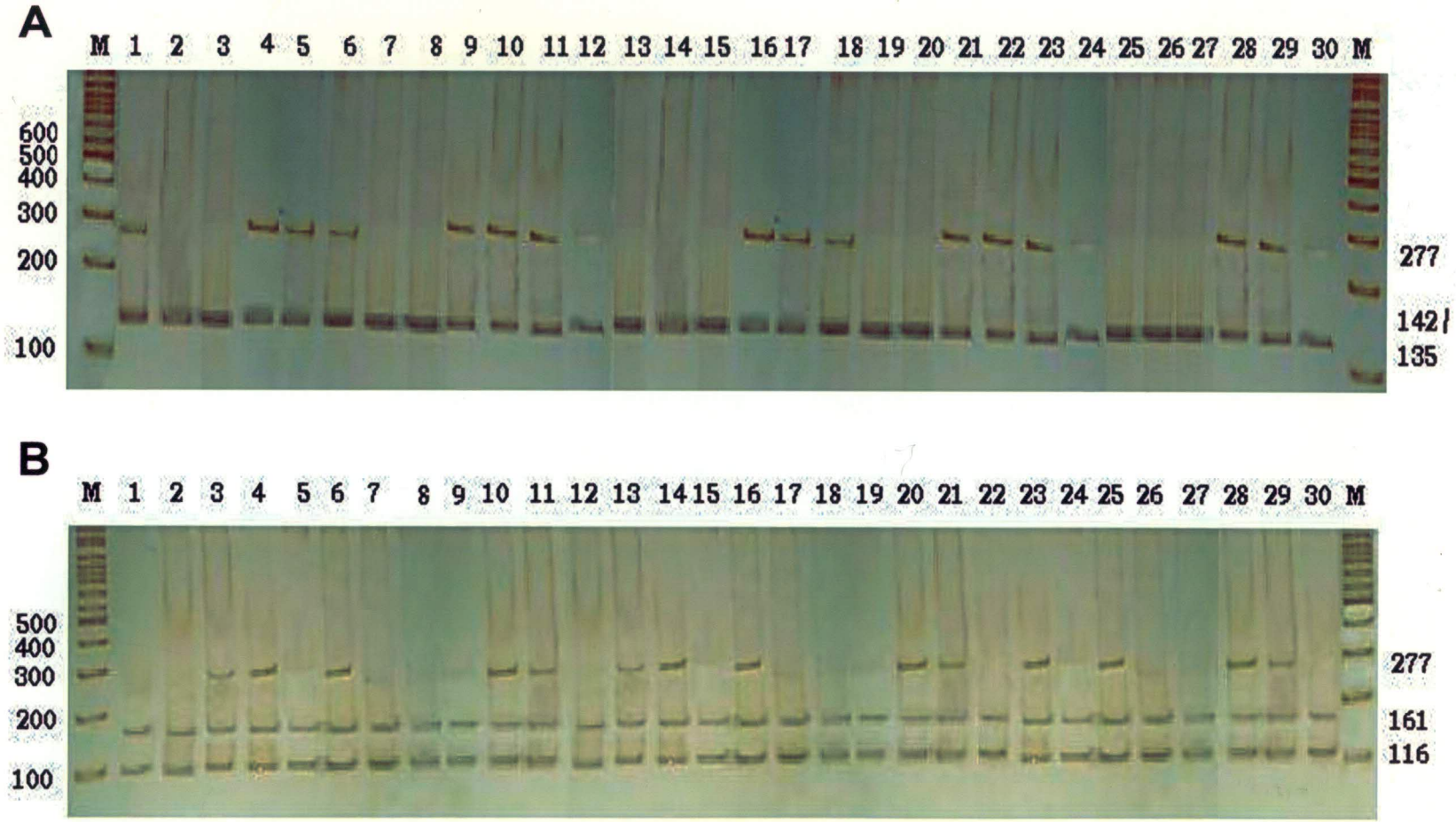


Fig. 4.6. PCR-RFLP pattern with *Hae III* (A) & *Taq I* (B) restriction enzymes in guinea fowl., 1 to 10 : Low HA titre; 11-20 : Medium HA titre l, 21-30 : High HA titre, M: Molecular size marker (100 bp ladder)

Table 4.1. Allelic frequencies at LEI 258 locus in guinea fowl & different chicken breeds.

S.No.	Allele size	GF	WL	AS	RC
1.	193	-	-	-	0.107
2.	205	-	0.033	-	0.357
3.	220	-	-	-	0.25
4.	234	-	0.133	0.133	0.035
5.	247	1.00	-	0.166	-
6.	249	-	0.066	0.1	-
7.	261	-	-	0.266	0.035
8.	274	-	-	-	0.178
9.	295	-	-	0.033	0.035
10.	321	-	-	0.033	-
11.	405	-	-	0.066	-
12.	420	-	-	0.066	-
13.	443	-	-	0.066	-
14.	552	-	-	0.033	-
15.	590	-	0.566	0.033	-
16.	608	-	0.2	-	-

Table 4.2. Possible B – haplotypes based on allele size at LEI 258 locus as per Fulton et al. 2006

S.No.	Allele size	Allele size as in Fulton et al. 2006	Possible B haplo-type as per Fulton et al. 2006	Remark
1.	193	193	11/15.1/27/61	-
2.	205	205	13/13.2/17/BW 11	
3.	220	-	Unidentifiable	New
4.	234	-	Unidentifiable	New
5.	247	247	18	-
6.	249	249	15.2/22/73	-
7.	261	261	2/15/29	-
8.	274	-	Unidentifiable	New
9.	295	295	5/11.1	-
10.	321	321	74	-
11.	405	405	1/1.2/8	-
12.	420	420	62	-
13.	443	443	6	-
14.	552	552	19.1	-
15.	590	-	Unidentifiable	New
16.	608	-	Unidentifiable	New

Table 4.3. Probable B haplotypes for LEI 258 locus in Guinea fowl & chicken breeds

S.No.	Type	Probable B haplotypes
1.	Guinea fowl	18
2.	White leghorn	13/13.2/15.2/17/22/73/BW 11/ Unidentifiable
3.	Aseel	1/1.2/2/5/6/8/11.1/15/15.2/18/19.1/22 /29/62/73/74/ Unidentifiable
4.	Red Cornish	2/5/11/11.1/13/13.2/15/15.1/17/27/29 /61/BW 11/ Unidentifiable

Table 4.4. SSCP Patterns in various titre groups of Guinea fowl.

S.No.	Type of pattern	Low antibody titre group	Medium antibody titre group	High antibody titre group	Total
1.	P - I	29,40	24,25 & 27	None	5
2.	P -II	None	35,50	2, 3, 41& 42	6
3.	P -III	14, 15, 51, 75 & 76	17, 19, 43, 49, 53, 54, 55, 58, 62, 66, 69 & 71	12, 70, 72 & 73	21
4.	P -IV	31	48, 56, 57, 59, 60, 64 & 65	4, 9, 10, 18 & 23	13
5.	P –V	7, 8, 20, 21, 22, 28 & 30	26, 32, 33, 34, 36, 37, 39 & 67	None	15
6.	P -VI	5, 6, 13 & 16	44, 47 & 52	11	8
	Total	19	35	14	68

Note: Numbers shown in different groups corresponding to the lane number shown in Figure 4.4.

Table 4.5. Proportion of various SSCP patterns in different titre groups of Guinea fowl.

S.No.	Type of Pattern	Frequencies of different patterns in different titre groups			Overall
		LHA	MHA	HHA	
1.	P – I	0.105	0.086	Nil	0.074
2.	P – II	Nil	0.057	0.285	0.088
3.	P – III	0.263	0.343	0.285	0.308
4.	P – IV	0.053	0.200	0.357	0.193
5.	P –V	0.368	0.228	Nil	0.220
6.	P –VI	0.211	0.086	0.073	0.117

Table 4.6. Genotypic frequencies of different genotypes in different HA titre groups in Guinea fowl population with different restriction enzymes.

Restriction enzymes/ HA antibody titre group	Genotype AA		Genotype AB	
	No.	Freq.	No.	Freq.
Hae III				
Low	4	0.4	6	0.6
Medium	6	0.6	4	0.4
High	5	0.5	5	0.5
Taq I				
Low	6	0.6	4	0.4
Medium	5	0.5	5	0.5
High	5	0.5	5	0.5

Discussion

Discussion

Present study was under taken with the objective of genotyping the guinea fowl population for B haplotyping using microsatellite based method (Fulton et al., 2006). Further, attempts were also made to study the polymorphism in MHC genes, targeting the BLB2 gene in guinea fowl using SSCP and PCR-RFLP techniques. Subsequently, attempts were made to establish an association between SSCP and PCR-RFLP pattern with general immunocompetence.

5.1. Development of resource population Assessment of general immune competence in guinea fowl

The general immuno-competence status of the 100 birds was assayed by measuring antibody response to SRBC through performing HA test at the age of 10 weeks of age. On the basis of HA antibody titre values, these birds were divided in to three groups viz. low HA antibody titre (LHA), medium HA antibody titre (MHA) and high HA antibody (HHA) titre groups. The mean HA antibody titre was 3.36 ± 0.11 in LHA group, 5.85 ± 0.11 in MHA group and 8.53 ± 0.19 in HHA group. These estimated values differ significantly (P>0.01) between the groups. The overall mean HA titre in guinea fowl was 5.74 ± 0.09 and was comparable with earlier reports in guinea fowl (Saxena *et al.*, 1997, Sharma and Singh, 2005, Sharma, 2007).

The antibody response against SRBC has been widely used to measure the general immunocompetence (Siegel and Gross, 1980, Ubosi *et al.*, 1985, Kundu *et al.*, 1999, Haunshi *et al.*, 2002, Khehn *et al.*, 2006). The divergent lines for immunocompetence traits (Siegel and Gross, 1980, Pitcovski *et al.*1989, Cheng and Lamont, 1990, Okabayashi and Okada, 1989) have widely been used as resource population for studying the antibody response to other antigens, cell-mediated immune responses, their interactions etc. (Pinard-van-der Laan *et al.* 1998, Lamont *et al.* 2003). Alternatively workers have also used the high HA titre group and Low HA titre groups made on the basis of the titre estimates within a population (Jackeray *et al.*, 2007, Raghuvanshi *et al.*, 2007). In this study we have used the groups differing for HA titres.

5.2. Genomic DNA isolation

A typical protocol to extract DNA from whole blood involves the lysis of nucleated blood cells and subsequent protein degradation by protinase K followed by organic solvent extraction. One of the time consuming steps in this procedure is the overnight protinase K treatment. This step has been reduced in the protocol adopted in present study to 6-7 hrs. We have observed that this modified protocol is highly reproducible and yields good quality genomic DNA. The high quality of genomic DNA isolated is evident from the resolution of DNA on 0.8 % agarose gel as single discrete band without any smear and RNA contamination. Further, the 260/280 nm absorbance ratio ranging from 1.7 to 1.9 for most of the samples indicated high quality intact DNA. The DNA samples showing absorbance ratio at 260 and 280 nm between 1.7 and 1.9 were taken for further analysis. The yield of genomic DNA in present study was estimated to be about 200-300 µg per 50 µl of blood. Similar yields of genomic DNA were reported by Hillel *et al.* (1989) and Sharma and Appa Rao (2000) in guinea fowl and different chicken breeds.

No differences were observed for yield of genomic DNA between the species i.e. Guinea fowl and chicken.

5.3. Microsatellite analysis

Microsatellites consist of tandem repeats of core sequences of di, tri, or tetra nucleotide units. The basis of polymorphism for a microsatellite locus lies in the fact that differences in number of the repeat motif (core sequences) generated by unequal crossover between the repeat units during meiosis among populations/ individuals. Hence the microsatellite locus having more number of alleles due to high polymorphism are more useful in determining genetic variation and phylogenies of organism, especially when these markers are used for studying the genetic diversity between populations of the same species (Vanhala *et al.*, 1998).

5.3.1. Microsatellite polymorphism

In present study, we have used one microsatellite marker i.e. LEI 258, which maps to chromosome 16 (McConnell *et al.*, 1999). This marker is also found in a clone that encompasses a large portion of the chicken MHC (GenBank AL023516; Guillemot *et al.*, 1988). In guinea fowl, only one allele i.e. 247 bp allele was found at LEI 258 locus; however in chicken breeds, number of alleles were 5 in White leghorn, 11 in Aseel and 7 in Red Cornish. Allelic size range was from 205 bp to 608 bp in White leghorn, 234 bp to 590 bp in Aseel and 193 bp to 295 bp in Red Cornish. Fulton *et al.* (2006) reported considerable greater size diversity at LEI 0258 locus in chicken. A total of 26 alleles, ranging from 182 to 552, were identified for LEI0258.

In general, number of alleles at a microsatellite locus depends upon the amount of heterozygocity present in the population. Most of the workers reported 3 to 5 alleles between the less diverged populations

and 5 to 15 between more diverged populations. Crooijmans *et al.* (1996) reported average number of marker alleles was 5.8 over all lines. Similarly, Kaiser *et al.* (2000) reported the average number of alleles per primer to be 2.8 and 2.9 in two chicken populations, while Vanhala *et al.* (1998) reported 4 to 13 alleles using nine different microsatellite markers with an average of 5.7 alleles per marker among the chicken lines of different genetic origin. Cheng *et al.* (2003), using five microsatellite markers with high polymorphisms estimated reported 5 (ADL0146) to 10 alleles (ADL0136). Hillel *et al* (2003) reported 4 to 23 alleles across the populations. Li *et al.* (2004), using 20 microsatellite markers in four package lines of egg-type chickens, reported 3.25 alleles / locus. Romanov and Weigend (2001) compared 20 chicken populations of different origin by typing 14 microsatellites and reported 2 and 21 alleles with the mean of 11.2 alleles per locus. Similarly, Qu *et al.* (2006) reported 6 to 51 alleles in 78 indigenous chicken breeds at 27 microsatellite loci. Average number of alleles was 18.74 per locus. Haunshi and Sharma (2006) reported 2 to 7 alleles with an average of 3.3 alleles per marker. Tomar *et al.* (2007) also reported 3 – 6 alleles in RC, AS, RJF and WL at 5 microsatellite loci. Muchadeyi *et al.* (2007) using 29 microsatellites and 13 indigenous populations of Zimbabwe, found 9.7 ± 5.10 alleles/locus.

Among chicken breeds 608 bp allele was found specific for WL with a frequency of 0.200, while the 193 bp allele with a frequency of 0.107, 220 bp allele with a frequency of 0.250 & 274 bp allele with a frequency of 0.178 were specific to RC. The numbers of specific alleles were 5 in AS i.e. 321 bp, 405 bp, 420 bp, 443 bp & 552 bp and all these alleles showed a low allelic frequencies ranging from 0.033 to 0.066.

The microsatellite markers are the potential markers for developing population specific profile as they show private alleles or population

specific alleles. Zhou and Lamont (1999) reported line-specific alleles among breeds and lines i.e. Leghorn, Jungle Fowl, Fayoumi and Spanish breeds. Hillel *et al.* (2003) reported 32 private alleles for the 52 chicken populations using 22 microsatellite markers. Wardecka *et al.* (2004) determined microsatellite polymorphism in Rhode Island Red (RIR) and Sussex (SX) chickens, divergently selected over six generations for high (H) or low (L) incidence of skeletal defects in embryos (30.7% for H lines, 3.7% for L lines). The polymorphism analysis covered 15 microsatellite markers within four lines (a total of 60 individuals). Eight alleles were identified as specific to H lines and six alleles as specific to L lines. Nakamura *et al.* (2006) used 25 microsatellite markers to identify the polymorphism between 4 strains of Nagoya breed (native to Japan) from other breeds and commercial stocks of chicken. In these strains, 5 of the markers (ABR0015, ABR0257, ABR0417, ABR0495, and ADL0262) had a single allele, while no other chicken breeds and hybrids had the same allele combination as the Nagoya breed strains. Hence, these 5 microsatellite markers provide a practical method to accurately discriminate the Nagoya breed from other chicken breeds. Rikimaru and Takahashi (2007) successfully discriminated the Hinai-jidori chicken from other chickens on the basis of Hinai-jidori specific alleles at 14 marker loci i.e. ABR1003, ADL0250, ABR0241, ABR0311, ABR1004, ABR1013, ABR0633, ABR1005, ABR0089, ABR1007, ABR1001, ABR1009, ABR1010, and ABR1011. Hence the microsatellite assay can effectively be used in discriminating a breed / line from other populations by identified population specific alleles.

5.3.2. Probable B-haplotypes in Guinea fowl and chicken breeds

In present study, our preliminary objective was of MHC haplotyping of the guinea fowl population based on allelic size at LEI 0258 locus as defined by Fulton *et al.* (2006). They have reported that most of the

standard B haplotypes could be distinguishable on the basis of LEI0258 allele size and this association between LEI0258 allele and serologically defined MHC haplotype was very consistent for the same haplotype from multiple sources. In guinea fowl, only one LEI0258 allele was observed i.e. 247 bp, which corresponded to B 18 haplotype as per Fulton *et al.* (2006), however this allele was also found in Aseel breed of chicken. The B 18 haplotype was reported in White Leghorn (UCD line) by Fulton *et al.* (2006) and has not studied well. The probable presence of this B haplotype in Guinea fowl might be an interesting finding and demans more detailed and in depth investigation.

In chicken breeds, a total of 16 alleles were found at LEI0258 locus. Out of these, 11 were those, which were also reported by Fulton *et al.* (2006), while 5 alleles were new. Out of the 11 alleles, only 5 i.e. 247 bp, 321 bp, 420 bp, 443 bp and 552 bp correspond to the specific B haplotypes i.e. B18, B74, B62, B6 and B 19.1, respectively, while others correspond to more than one B haplotypes and these haplotypes are further distinguishable on the basis of the MCW0371 allele size.

Fulton *et al.* (2006) reported that the most common allele size was 357, which was found in ten different haplotypes, originating from six different breed sources. The most well-defined haplotype with the 357 allele is B21. The B21 haplotype is known to confer resistance to MDV (Hansen *et al.* 1967; Briles *et al.* 1977; Bacon *et al.* 1981). However this allele was not found in our chicken populations. Another widely dispersed allele was 193 bp, being found in four different haplotypes (including B11), representing three different breeds. It is interesting that the B11 haplotype is also known to confer resistance to MDV (Wakenell *et al.* 1996). In our chicken populations, it was found only in RC.

The 590 bp and 608 bp allele was predominantly present in our WL population with a cumulative frequency of 0.766, but this allele was not reported by Fulton *et al.* (2006). Similarly, in our RC population, the

predominantly occurring alleles were 193 bp, 205 bp, 220 bp and 274 bp, showing a cumulative frequency of 0.892. Among these, only two i.e. 193 bp and 205 bp were reported by Fulton *et al.* (2006) and corresponded to B11 / B 15.1 / B 27 / B 61 and B13/ B13.2/ B17/ BW11, respectively, while other two were not reported earlier. In our Aseel population, the predominantly present alleles were 234 bp, 247 bp, 249 bp and 261 bp, showing a cumulative frequency of 0.665. Among these, three alleles i.e. 247 bp, 249 bp and 261 bp were also reported by Fulton et al. (2006) and corresponded to B18; B15.2/ B22 / B73 and B2/ B15/ B29, respectively. The five haplotypes i.e. B18, B74, B62, B6 and B 19.1, respectively were found in Aseel only. Another Aseel specific allele was 405 bp, which corresponds to B1/ B1.2 / B8. Though all these haplotypes showed very low frequency, but might be important in view of better diseases resistance of Aseel.

5.4. SSCP analysis

In view of monomorphic pattern at LEI0258 locus, an attempt was made to explore the possibilities B haplotyping in guinea fowl based on SSCP pattern for BLB2 gene as suggested by Goto *et al.* (2002). The BLB2 gene is one of the predominantly expressed MHC gene in chicken. The β1 domain of BLB2 gene is highly polymorphic (Zoorob *et al.* 1990; Sung *et al.* 1993; Singh *et al.* 2005).Hence, the SSCP technique was used to detect the polymorphism in β1 domain of BLB2 gene in guinea fowl and to establish an association between SSCP pattern and antibody response to SRBC.

The 277 bp fragments representing β1 domain of BLB2 gene amplified from all the 76 GF birds were resolved on 10 % native polyacrylamide gel & the gels were stained with ethidium bromide and photographed under white light. Various SSCP patterns were observed

depending upon the mobility of secondary structures formed by the single strands in non denaturing gel. In guinea fowl, total of 6 SSCP patterns were observed (Fig 4.4) and out of these, Pattern III was predominantly present (0.308), followed by pattern V (0.220) and pattern IV (0.193).

Goto *et al.* (2002) have developed a DNA-based method for defining MHC B system genotypes in chickens. Allelic differences at chicken B-F (class I) and B-L (class II) loci are detected in PCR single-strand conformation polymorphism (SSCP) assays. PCR primer pairs were designed to hybridize specifically with conserved sequences surrounding hypervariable regions within the two class I and two class II loci of the B-complex and used to generate DNA fragments that are heat- and formamide denatured and then analyzed on nondenaturing polyacrylamide gels. Typing is accomplished by comparing SSCP patterns representing haplotypes rather than individual bands. Concordance was found between SSCP patterns and previously assigned MHC types. They studied the B-L SSCP patterns in 20 randomly selected individuals within the SPAFAS line 22 in which at-least four B haplotypes known from serological typing were segregating. Seven B-L SSCP patterns were found among the 20 samples. Hence, the present result suggests that in guinea fowl, at least more than one B haplotype exist.

Among the titres groups, some specific trend was observed. The pattern V was predominantly present in LHA and MHA groups, while completely absent in HHA group. Similarly, pattern IV showed maximum frequency in HHA (0.357), followed by MHA (0.200) and very low in LHA (0.053). The pattern III showed good frequency in all the groups (0.263 to 0.343). These preliminary trends are encouraging, but demands further investigations.

5.5. PCR RFLP studies in BLB2 gene in guinea fowl

The class II molecules of MHC include the α and β chains of the heterodimeric peptide binding proteins and among them, β chain gene, especially the peptide-binding region (PBR) in β1 domain is polymorphic (Jacob *et al.*, 2000). The nucleotide sequence variability in exon 2 region of BLB2 used to create or abolish the sites for various restriction enzymes, thus provide a simple and effective way to detect polymorphism in population by PCR-RFLP of a specific genomic region. Two restriction enzymes i.e. *Hae* III and *Taq* I were selected to detect polymorphism in this region among the groups differing for response to SRBC in guinea fowl. Both the restriction enzymes showed polymorphism between the groups. PCR-RFLP was also observed between White Leghorn and RJF for Pst I restriction site in similar region and higher hetrozygocity was observed in White Leghorn in comparison to RJF (Singh, 2005) however, Ahmed *et al.*, (2007) also reported monomorphic PCR-RFLP profile of similar region i.e. exon 2 of BLB2 gene in turkey.

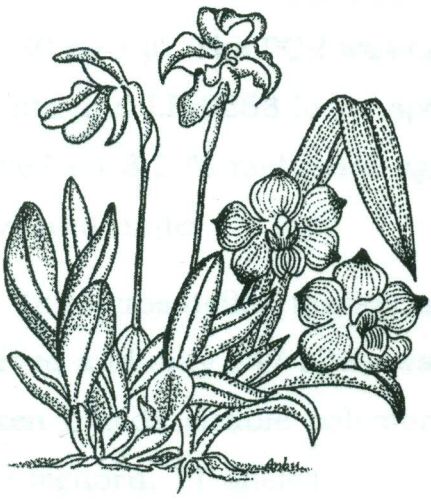
Based on *Hae* III RE profiles, frequencies of AA & AB genotypes in different antibody titre groups in guinea fowl were 0.4 and 0.6 in low titre group, 0.6 and 0.4 in medium titre group and in 0.5 & 0.5 in high titre group respectively. Similarly, based on Taq I RE profiles, the frequencies of AA & AB genotypes in different antibody titre groups were 0.6 and 0.4 in low titre group, 0.5 and 0.5 in medium titre group and in 0.5 & 0.5 in high titre group respectively. These results suggested no trend between the RE profiles and response to SRBC, inspite of ample polymorphism between as well as with the titre groups.

Ouyang-jianhua *et al.* (2000) showed that polymorphism detected by EcoRV and Xha I through PCR-RFLP were not related to egg production and fertility rate. However, Ewald *et al.* (2007) reported significant BF2

associations with a subset of traits were observed in two commercial broiler lines. The BF2*21 allele was positively associated with antibody titre to infectious bursal disease virus in both lines. Other associations were line-specific.

Ahmed (2001), using *Taq* I and *Hae* III restriction enzymes, could not get the polymorphism between the divergent lines for antibody response to SRBC as well as CMI to mitogens. Shivakumar (2003) studied the polymorphism in the âI exon of the BL B II gene in the divergent lines for response to sheep red blood cell in white leghorn chicken. However, using *Taq* I restriction enzyme, he could not get the polymorphism between high titer line and low titer line in IWG as well as in IWJ lines of White Leghorn. Similarly, Muthukumar (2003) studied the 2nd generation of HSRBC, LSRBC, HCMI and LCMI lines in broiler. He studied polymorphism using PCR-RFLP between these lines, however using *Taq* I; he also could not get polymorphism between these lines.

Summary and Conclusions



Summary and Conclusions

Present study was under taken with the objective of genotyping the guinea fowl population for B haplotyping based on microsatellite based method. Further, attempts were also made to study the polymorphism in MHC genes, targeting the β1 domain of BLB2 gene in guinea fowl using SSCP and PCR-RFLP techniques. Attempts were also made to establish an association between SSCP and PCR-RFLP pattern with general immunocompetence.

For B haplotyping based on LEI 0258 allele size, a total of 76 guinea fowl birds of Lavender variety (GF) and 15 birds from each of the three chicken breeds i.e. White Leghorn (WL), Aseel (AS) and Red Cornish (RC) were used. The genomic DNA was extracted using phenol chloroform extraction method. The samples were further diluted to the concentration of 25-30 ng / µl. The PCR was carried out using these DNA samples as template and LEI0258 locus specific primers. The PCR products were resolved on 3.5 % metaphor agarose, stained with ethidium bromide and viewed under UV light.

In microsatellite marker study in GF monomorphic pattern was observed and only 247 allele was found for LEI 258 locus; however in chicken breeds sizable polymorphism was observed with 5 alleles in White leghorn, 11 alleles in Aseel and 7 alleles in Red Cornish. Allelic

size range was from 205 to 608 bp in White leghorn, 234 to 590 bp in Aseel and 193 to 295 bp in Red Cornish. Among chicken breeds 608 bp allele in WL, 193 bp, 220 bp & 274 bp alleles in RC & 321 bp, 405 bp, 420 bp, 443 bp & 552 bp in AS were found to be specific with allelic frequencies ranging from 0.033 to 0.25. The 247 bp allele which was fixed in GF also found in AS though at a very low frequency of 0.166.

Possible B haplotypes were predicted in guinea fowl as well as chicken populations. In GF only one B-haplotype i.e. B18 was observed, however in chicken breeds several B-haplotypes were observed. In WL, the observed B-haplotypes were 13, 13.2, 15.2, 17, 22, 73 and BW11, while in AS B-haplotypes 1, 1.2, 2, 5, 6, 8, 11.1, 15, 15.2, 18, 19.1, 22, 29, 62, 73 & 74 were observed. In RC, the observed B- haplotypes were 2, 5, 11, 11.1, 13, 13.2, 15, 15.1, 17, 27, 29, 61 & BW11. Among all observed B-haplotyes in chicken breeds, haplotypes 1, 1.2, 19.1, 62 & 74 were found only in AS, whereas haplotype 15.1, 27 & 61 were found only in RC. None of the haplotype was found which was present only in WL.

In view of monomorphic pattern at LEI0258 locus, an attempt was made to explore the possibilities B haplotyping in guinea fowl based on SSCP pattern for BLB2 gene as suggested by Goto and workers. A 277 bp fragment representing β1 domain of BLB 2 gene was successfully amplified in guinea fowl population. The PCR product was resolved on 10 % native PAGE and silver stained for SSCP analysis. A total of 6 SSCP patterns were observed and out of these, Pattern III was predominantly present (0.308), followed by pattern V (0.220) and pattern IV (0.193). Presence of 6 SSCP patterns suggested the possibilities of more than one B haplotype in guinea fowl.

The present resource population of guinea fowl was grouped on the basis of total HA titre against SRBC at 10th weeks of age. Three

groups viz. low HA antibody titre (LHA), medium HA antibody titre (MHA) and high HA antibody (HHA) titre groups were made. The mean HA titre was 3.36±0.11 in LHA group, 5.85±0.11 in MHA group and 8.53±0.19 in HHA group. These estimated values differ significantly (P>0.01) between the groups. Among the titres groups, some specific trend was observed. The pattern V was predominantly present in LHA and MHA groups, while completely absent in HHA group. Similarly, pattern IV showed maximum frequency in HHA (0.357), followed by MHA (0.200) and very low in LHA (0.053). The pattern III showed good frequency in all the groups (0.263 to 0.343). These preliminary trends are encouraging, but demands further investigations.

The polymorphism in BLB 2 gene by targeting the β1 domain of BLB 2 gene through RFLP technique was also studied in guinea fowl. Two restriction enzymes i.e. Hae III & Taq I were selected for study. The 277 bp amplified fragment from 30 individuals genomic DNA (10 each from LHA, MHA & HHA groups) were digested with these restriction enzymes and the digested products were resolved on 6 % native polyacrylamide gel, silver stained and viewd under UV light. In present study with *Hae III* restriction enzyme, two alleles i.e. Allele A (having RE site at 142 nt) and allele B (not having RE site) and two genotypes i.e. genotype AA (homozygous for presence of RE site) and AB (heterozygous for presence of RE site) were observed. The frequency of these two i.e. AA & AB genotypes in different antibody titre groups in guinea fowl was 0.4 and 0.6 in low titre group, 0.6 and 0.4 in medium titre group and in 0.5 & 0.5 in high titre group respectively. Similarly with *Taq I* restriction enzyme, two alleles i.e. Allele A (having RE site at 116 nt) and allele B (not having RE site) and two genotypes i.e. genotype AA (homozygous for presence of RE site) and AB (heterozygous for presence of RE site)

were observed. The frequency of these two i.e. AA & AB genotypes in different antibody titre groups in guinea fowl was 0.6 and 0.4 in low titre group, 0.5 and 0.5 in medium titre group and in 0.5 & 0.5 in high titre group respectively. No association could be observed between RE profiles and HA titre against SRBC.

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Annexure

Molecular Genetic Studies

1. Isolation of genomic DNA

PBS (Phosphate buffered saline; pH 7.2 – 7.4)

NaCl	8 gm
KCl	2 gm
Na_2HPO_4	1.44 gm
KH_2PO_4	0.24 gm

Dissolve in 800 ml of auto D.W., adjust pH to 7.2 – 7.4 with HCl and make volume to 100 ml. Sterilize by autoclave. Store at 4°C.

Lysis buffer (pH 8.0)

2M Tris HCl	2.5 ml
0.4M EDTA	6.25 ml
2M NaCl	5.0 ml

Auto D.W. upto 100 ml. Store at 4°C.

10% SDS : Sodium dodecyl sulphate

Sodium dodecyl sulphate	10 gm
Auto D.W.	100 ml

Store at room temperature. Heat at 60°C before use.

5M NaCl

NaCl	292.9 gm
Auto D.W.	1000 ml

Autoclave and Store at 4°C.

2M Tris

Tris	242.2 gm

Dissolve in 800 ml of Auto D.W. Adjust the pH 68.0 by HCl. Make volume up to 1000 ml. Autoclave and store at 4°C.

0.4M EDTA (pH 8.)

EDTA	148.8 gm

Dissolve in 500 ml of auto D.W. with the help of magnetic stirrer for 1-2 hours and adjust the pH to 8.0 by NaOH pellets. Make the volume upto 1000 ml, sterilize by autoclave. Store at 4°C.

1X TE (Tris : EDTA: 10:1)

2M Tris	125 µl
0.4 M EDTA	62.5 µl

Auto DW upto 25 ml.

Chloroform:Isoamyl alcohol (24:1)

Chloroform	24 ml
Isoamyl alcohol	1 ml

Mix thoroughly and store at 4°C.

Phenol:Chloroform:Isoamyl alcohol.

Tris saturated phenol	25 ml
Chloroform isoamyl alcohol	25 ml

Mix thoroughly and store at 4°C.

Tris-saturated phenol

a) Melt phenol at 68°C by keeping in water bath.

b) Measure the required volume. Add 8-hydroxy quinoline to a final concentration of 0.1% (It is an anti-oxidant, gives yellow colour to phenol).

c) Extract phenol several times with equal volume of 1M Tris (pH 8.0).

d) Then, with 0.1M Tris, until the pH of the aqueous phase is more than 7.6.

e) Add 0.2% D-mercaptoethanol

f) Mix thoroughly and store in amber-coloured bottle at 4°C.

Proteinase K (5 mg/ml)

Proteinase K	5 mg
Auto DW	1ml

Store at –20°C.

2. Setting up of PCR

10X Taq buffer

Tris-HCl (pH 8.8)	100 mM
KCl	500 mM
$MgCl_2$	15 mM
Triton X-100	1%

Store at –20°C.

Taq DNA polymerase enzyme

Taq DNA polymerase	5 units/µl

Store at –20°C.

dNTP solution (pH 7.0)

dATP	10 mM
dCTP	10 mM
dGTP	10 mM
dTTP	10 mM

Store at –20°C.

Primers

Stock: 200 µg dissolved in 100 µl.

Add 2 µl from stock in 198.23 µl of auto DW. Use 2 µl (40 mg) per PCR mixture.

3. Gel Electrophoresis (Agarose Gel Electrophoresis)

5X Tris Borate EDTA (TBE)

Tris base	54 gm
Boric acid	27.5 gm
0.4M EDTA (pH 8.0)	25 ml

Auto DW upto 1000 ml and Sterilize by autoclave. Store at room temperature.

50X Tris Acetate EDTA (TAE)

Tris base	24.2 gm
Glacial acetic acid	5.71 ml
0.4M EDTA (pH 8.0)	25 ml

Auto DW upto 100 ml and Store at room temperature.

6X Gel Loading Due

Bromophenol blue	0.25 %
Sucrose	40% (W/V)

Store at 4°C.

20,000X Ethidium Bromide (10 mg/ml)

Ethidium bromide	10 mg
Auto DW	1 ml

Wrap in an aluminium foil (Photosensitive). Store in a dark place at room temperature.

Molecular Weight Marker

a) 100 bp- 10 fragments of 1000, 900, 800, 700, 600, 500, 400, 300, 200 and 100 bp.

4. 6% Native Polyacrylamide Gel Electrophoresis (PAGE)

30% Acrylamide solution

Acrylamide	29gm
Bisacrylamide	1gm
Distilled water	50ml

Make volume upto 100ml. kept at 20°C.

Ammonium Persulphate solution (APS)

Ammonium persulphate	0.1gm
Distilled water	1ml

Polyacrylamide gel preparation

30% Acrylamide solution	10ml
5X TBE	6ml
DistilledWater	14ml

10% APS	260ml
TEMED	12ml

5. Silver Staining

10% Alcohol preparation

Alcohol	20ml
Distilled water	180ml

1% HNO_3 solution

HNO_3	2ml
Distilled water	198ml

Silver nitrate solution

Silver nitrate ($AgNO_3$)	0.45gm
Distilled water	300ml

Developer solution

Sodium Carbonate (Na_2CO_3)	15gm
Distilled water	500ml
Formaldehyde (HCHO)	250ml

Stopper solution

Glacial Acetic acid	20ml
Distilled water	30ml

6. Immunological Studies

1. Haemagglutination (HA) Titre

PBS See in molecular genetic studies

2. Normal saline solution (NSS)

8.5 g of sodium chloride (NaCl) in 1000 ml of distilled water. Autoclaved at 15 lbs pressure for 20 min.

3. Phosphate buffer saline (PBS, pH 7.4)

Sodium chloride (NaCl)	8.00 g
Potassium dihydrogen phosphate (KH_2PO_4)	0.20 g
Disodium hydrogen phosphate ($Na_2HPO_42H_2O$)	1.16 g
Potassium chloride (KCl)	0.20 g
Distilled water	1000 ml

The solution was autoclaved at 121°C, 15 lbs pressure for 30 min and stored at 4°C for further use.

4. Alsever's solution

Dextrose (BDH)	20.5 g
Sodium citrate (BDH)	8.0 g
Citric acid (BDH)	0.55 g
Sodium chloride	4.2 g
Distilled water	1000 ml

The pH adjusted to 6.1 with citric acid solution.

Autoclaved 10 lbs pressure for 15 min and stored at 4°C.