STUDY OF IMMUNOLOGICAL STATUS OF NATIVE CHICKENS

Thesis submitted in partial fulfillment for the award of Degree of Doctor of Philosophy in
BIOTECHNOLOGY

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DECLARATION

I RAJNI SAXENA declare that the thesis entitled “STUDY OF IMMUNOLOGICAL STATUS OF NATIVE CHICKENS” submitted by me for the Degree of Doctor of Philosophy in Biotechnology is the record of work carried out by me during the period from October 2008-2014 under the guidance of DR. R. STEPHAN, M.Sc., M.C.A., M.Tech., PhD., and has not formed the basis for the award of any Degree, Diploma, associateship, fellowship, titles in this or any other university or other similar institutions of higher learning.

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I DR. R. STEPHAN, M.Sc., M.C.A., M.Tech., PhD., Certify that the thesis entitled “STUDY OF IMMUNOLOGICAL STATUS OF NATIVE CHICKENS” submitted for the degree of Doctor of Philosophy in Biotechnology by Ms. Rajni Saxena is the record of research of work carried out by him during the period from 2008-2014 under my guidance and supervision and that this work has not formed the basis for the award of any degree, diploma associateship, fellowship or other titles in this university or any other university or institutions of higher learning.

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(RAJNI SAXENA)
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India’s poultry industry has transformed from a mere backyard activity into a major commercial activity in just four decades. Poultry industry has made tremendous growth during last three decades and it has expanded multifold. Commercial strains of poultry have attained almost the plateau of production traits because of maximum exploitation of available genetic variations; hence further improvement in production performance through conventional breeding seems to be impractical.

Poultry health is an important factor as it not only reduces the losses due to morbidity and mortality but also influences the overall performance of the flock. Diseases are the biggest challenge for the poultry industry in Asian subcontinent. Diseases inflict a great economic loss (8% to 10%) to poultry industry (Bootwala, 2005).

Although proper vaccination, prophylactic and therapeutic medications and sanitation have reduced the incidence rate of diseases, poultry flocks continue to be plagued with diseases. Poultry is considered to be the best experimental animal because of its small size, shorter generation interval, low feed intake, easy handling and fast metabolism. Eggs and poultry meat are protective food and are the cheapest source of quality animal protein and have wide acceptability in India. The genetic resistance in poultry is controlled through immune
system that plays a dynamic role in health protection both genetic and environmental factors decide the execution of this role in the bird (Gavora, 1993). Other approach to control diseases includes the induction of immunity by vaccination. Moreover, it is apparent that use of improved vaccine is associated with the evolution of virulence of many pathogenic agents leading to increased disease losses until new generation of vaccines are introduced.

Aseel is one of the important indigenous chicken breeds of India. It is known for its pugnacity, high stamina, and majestic gait and dogged fighting qualities. The best specimens of the breed, although rare, are encountered in parts of Andhra Pradesh, Utter Pradesh and Rajasthan. The most popular varieties are Peela (Golden Red), Yakub (Black and Red), Nurie (White), Kagar (Black), Chitta (Black and White spotted), Java (Black), Sabja (White and Golden or Black with Yellow or Silver), Teekar (Brown) and Reza (Light Red). In Aseel, genetic resistance has been found more than any other Indian native breed and significantly higher serum IgG level than White Leghorn (Ahrestani et al., 1987). The Central Avian Research Institute (CARI) reports 92 eggs per annum from Aseel with an average egg weight of 52 g (www.icar.org/cari/native.html) vs. 33 eggs per annum from unimproved Aseel Chicken in Bangladesh (Huque et al., 1999; Bhuyian et al., 2005). Immunocompetence is the ability of the body to produce a
normal response following exposure to an antigen. Immunocompetence is the opposite of immuno-deficiency or immuno-incompetent. Immunocompetence in poultry can be evaluated by challenging the birds with antigens. These antigens used to challenge birds may be causative agent of diseases including Marek’s disease virus or New Castle disease virus or some other causative agents of diseases, but disadvantage is that they are expensive, time consuming and will cause morbidity and mortality in flock due to their pathogenic nature. So the appropriate way to study immunocompetence in a species is to challenge it with nonpathogenic, non-specific antigen like Sheep Red Blood Cells (SRBC), Mollusk Haemocyanin, Chicken Egg White Lysozyme and Bacterial lipo-polysaccharides but among all these SRBC is the most commonly used antigen to study immuno-competence in poultry (Vanderzipp, 1983). (Norris & Evans, 2000) defined immunocompetence as the ability of a host to prevent or control infection by pathogens and parasites, and variation in immunocompetence is assumed to represent general individual disease resistance.

Several immunocompetence traits that can be considered for improving genetic resistance to diseases in poultry are greater antibody response to sheep RBC, lysozyme activity and high titer of immunoglobulin G (IgG) in the serum. Antibody titers against sheep RBC and serum IgG level are the indicators of humoral immune response,
whereas bacteriolytic activity of serum lysozyme is the indicator of non-specific immune response.

In the present study Aseel (n = 301), an Indian breed of chicken, was studied for high and low immune response by assessing their immunocompetence traits using 3 different tests - hemagglutination (HA) test, lysozyme plate assay and serum IgG level estimation.

Cytokines regulate the intensity and duration of the immune response by stimulating or inhibiting activation, proliferation, or differentiation of various cells and regulating the secretion of antibodies or other cytokines. Interleukins form an important group of cytokines which play significant role in eliciting immune responses. IL-2 and IL-15 help in recruiting T-cells to tissues during immune response, proliferation of T-cells and Natural killer cells, growth stimulation of B-cells and immunoglobulin synthesis (Kumaki et al., 1996). IL-2 induces the proliferation and differentiation of T-cells, B-cells and NK cells (Kuby, 1997). The IL-2R plays a pivotal role in the formation of complete IL-2 receptor and cause human X-linked severe combined immunodeficiency, resulting in complete or profound T-cell defect (Ohbo et al., 1995). Molecular techniques have been found to be the powerful tools in analyzing the heterogeneity in the disease resistant status of poultry flock (Soller and Beckman, 1986).
Recent advancements made in the molecular genetics have given modern tools to study nucleotide polymorphism which include PCR - RFLP, SSCP etc. PCR - RFLP is one of the most popular techniques to identify candidate gene polymorphism (Beckman and Sollers, 1983). Polymerase Chain Reaction – Restriction Fragment Length Polymorphism (PCR - RFLP) assay is used to determine the genotype of chickens for various genes (Kramer et al., 2003). It has also been used to characterize interleukin gene of Aseel native chicken (Kumar, 2006). Occurrence of highest humoral response to SRBC has been recorded in chickens of Indian breed of different ecotypes subjected to non-pathogenic multideterminant SRBC (Baelmans et al., 2005) which signifies that our Indian breeds are of higher immunocompetence status than exotic breed.
The birds immune system is a complex network of specialized organs, glands and cells which when working properly protect the body from pathogens such as virus, bacteria and fungus. The immunocompetence status of any breed speaks about its general response to diseases. Resistance to diseases is under the control of certain immune response genes. Nucleotide polymorphism in such genes results in varied response of individuals to variety of pathogens/antigens. DNA polymorphism also has association with the nature of antibody response. The available literature on the proposed objectives has been reviewed below.

2.1. Evaluation of Immunocompetence status

The immunocompetence status can be evaluated by assessing important parameters related to various facets of immunity such as antibody response to SRBC, serum Lysozyme activity and serum IgG level etc. Various genetic groups/ varieties/breeds/species have also shown significant differences in antibody titres against SRBC, serum lysozyme level and serum immunoglobulin (IgG) level (Toro et al., 1997; Saxena, 1993; Saxena et al., 1997; Sarker et al., 1998; Santosh, 1999; Shivakumar, 2003; Singh et al., 2003; Singh, 2005).
(Heckert et al., 2002) Lymphoid organ weights are easily measured and reflect body's ability to provide lymphoid cells during an immune response. (Zhang et al., 2006 and Cheema et al., 2007) Reported that The bursa of Fabricius is a key lymphoid organ that is responsible for the development and maturation of B-lymphocytes, and the humoral antibody response is dependent on this central organ. 

(Van der Zijpp., 1983) reported that the capacity of antibody formation is inherited as a dominant trait some of the important parameters for assessment of immunocompetence have been reviewed.

2.1.1. Antibody response to SRBC

The antibody response is a part of the acquired immune system, which is produced by B cells and mediated by Th2 cells in response to antigenic stimulation (Janeway et al., 2005). The antibody response in young chicks is immature, but a large range of responsiveness has been observed. Chicks generally respond faster and more strongly with increasing age (Hatkin et al., 1993), which is probably, among other factors, related to the development of the immune system (O’Neill et al., 2006). 

The immune response to non-specific, natural, multi-determinant, complex and non-pathogenic antigens like sheep red blood cells (SRBC) may provide good indication of natural immunocompetency. The response to sheep red blood cells is T-cell dependent and has
association with B-G region of major histocompatibility. 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; Kuehn et al., 2006).

Sheep red blood cell (SRBC) is a non-pathogenic, T-cell dependent, non-specific, natural multideterminant antigen. It provides good indication of innate humoral immune response status of an individual because of its broad immune response characteristics, thus valid for multiple pathogens. The use of antibody responses to SRBC in a multitrait selection programme is a well-known concept in avian immunology that reveals various aspects of immune responses and their genetic basis. (Kean et al., 1994a).

The immunocompetence of young and immature vertebrates, including chicken, has been described as gradually increasing with age until maturity, when a given plateau is reached (Siegrist, 2001; Reese et al., 2006).

Immunocompetence and growth are influenced by genetic and non-genetic factors. There is evidence in the literature regarding negatively correlation between growth and anti-SRBC antibody response in Leghorn (Siegel et al., 1982), broilers (Qureshi and Havenstein, 1994), brown egg-layers (Kruekniet et al., 1994) and Egyptian native breeds (Yakoub et al., 2005).
Several workers have studied different immunocompetence traits in selection experiments based on immune response to a single antigen such as Sheep red blood cells (SRBC) in chicken (Gross et al., 1980; Van der Zijpp, 1983; Dunnington et al., 1984; Van der Zijpp et al., 1986; Scott et al., 1988; Pinard et al., 1992; Boa-Amponsem et al., 1997, and 2000; Shivakumar 2005; and Sivaraman 2004). Information regarding the immune status of native chicken may be obtained reliably and quickly by immunizing birds with sheep red blood cells (Kundu et al., 1999) to test specific humoral immunity and measuring complement activity as an innate immune response to SRBC (Haunshi et al., 2002).

(Seigel and Gross., 1980) Observed that Bi-directional selection experiment based on antibody response to Sheep RBC in Cornell random bred chicken and their crosses were carried out. The response to selection was immediate as highly significant differences in the titre values between the High and Low SRBC lines were observed. The response in first generation was 1.27 for males and 1.21 for females in first generation and progressively increased to 1.77 and 2.42 in second generation. They observed that the mean 5 dpi antibody titres of the crosses intermediate of the parental lines were significantly greater than the mid parent values and suggested the influence of non-additive gene action on antibody titres. They also observed the response to selection
for persistence and non-persistence of antibodies at 5 dpi and 21 dpi in the same population is highly significant between the lines.


(Norris & Evans, 2000) defined immunocompetence as the ability of a host to prevent or control infection by pathogens and parasites, and variation in immunocompetence is assumed to represent general individual disease resistance.

(Van der Zijpp, 1983) evaluated humoral immune response to SRBC in White Plymoth Rock and White Leghorn (WL) chickens, with emphasis on dose, genetic origin, interaction and correlation between primary and secondary immune responses and also between humoral and cell-mediated immune responses. They demonstrated significant differences in total antibody titers among genetic groups.

Recent studies have found contrasting results: in some avian species different components of immunity showed correlated responses (Westneat et al. 2003; Ekblom et al. 2005) whereas in others they did not (Zuk & Johnsen 1998; Faivre et al. 2003).

(Ubosi et al., 1985) analyzed the age dependency of antibody response to SRBC in divergently selected lines for this trait. There were differences among populations for frequency of responders at 7 days of
age. The serological maturity was reached at 14 days of age. After the attainment of serological maturity, the high SRBC line had significantly higher antibody titer than low line and the reciprocal crosses were intermediate to their parental lines. The weight of bursa and spleen was more in the high line and the thymus weight was less than those in low line.

(Gross, 1986) compared the doses required to get optimum response in the divergently selected lines for SRBC response and reported that the high line responded with low dosages of SRBC, whereas low line could not respond to that dose.

(Van der Zijpp and Nieuwland 1986) reported that in ISA Warren fowl line, selection for high and low anti-SRBC titer did not affect the cell-mediated immunity.

(Kim et al., 1987) estimated the correlation between the immunological and production traits in S1 White Leghorn line. The correlations (0.21-0.31) among response to SRBC and body weights in all the ages were significant.

(Scott et al., 1988) studied immune response to SRBC in small and large bursal chicken lines and its base population and revealed that $B_1B_1$ chicks produced significantly higher peak titre against SRBC than $B_6B_6$ chicks. In adults, the order of response for different genotypes was $B_1B_1$, $B_6B_6$ and $B_5B_5$. 
(Kreukniet et al., 1990) studied the effect of SRBC dosage on the humoral responses of chicken lines selected for high (H) and low (L) antibody production against SRBC and observed that the H line had higher and early peak titer response than L line after immunization. Further, the response was higher for pullets than cockerels in H line.

(Martin et al., 1990) studied the correlation between antibody titre, growth and reproduction traits in lines selected for high and low response to SRBC. The genetic correlations were moderate among most of the traits. They observed that the females of low lines for SRBC response were heavier and had high egg number as compared to those of high line.

(Pinard et al., 1990) estimated the $h^2$ for SRBC response in fowl selected divergently for 8 generations. The estimates of the realized heritability for response to SRBC in high line were 0.15 and 0.16 when estimated by regression and animal model, respectively. The corresponding values for low line were 0.22 and 0.26.

(Chao and Lee, 1991) reported higher immune response to SRBC in country chicken than White Leghorn in Taiwan.

(Miller et al., 1992) reported that additive genetic variation was important in inheritance of both primary and secondary response to antigen. Reciprocal differences and heterosis also influenced the secondary response. (Boa-Amponsen et al., 1997) measured SRBC
antibody titre in embryos and chicks of high and low SRBC lines. Maternal antibody was detected earlier in high line as compared to low line chicks.

(Kundu, 1997) studied SRBC response in Aseel, Kadaknath, Naked neck, Frizzle, Dahlem Red, White Leghorn, SDL broilers and Naked Neck broilers and revealed that all genetic groups showed highest titre on 5dpi except broilers which revealed peak titre on 12 days post immunization (dpi).

(Saxena et al., 1997) estimated the response to SRBC in Guinea fowl, Kadaknath and broilers as 1.520±0.487, 1.525±0.068, and 1.386±0.119, respectively. Significant differences among varieties and sire families were observed in guinea fowl.

(Santosh, 1999) evaluated the response to SRBC in White Leghorn, Aseel, Kadaknath, and Dahlem Red and their crosses, and reported significant genotype differences for SRBC response.

(Boa-Amponsem et al., 2000) studied the primary and secondary antibody responses to different dosages of SRBC, at different days of inoculation, in divergently selected lines for SRBC response. They observed different patterns of antibody response to SRBC dosages in different lines, which revealed interactions between line x dosage x days.
(Ahmed, 2001) observed in broiler chicken, developed divergent lines based on antibody response to SRBC at Central Avian Research Institute, Izatnagar. Significant differences in the antibody titre even after only one generation of selection were observed between lines.

(Shivakumar, 2005) estimated humoral immune response in divergently selected (high & low antibody response) IWG & IWJ lines of WL chickens and observed that the SRBC response was higher in high line than in low line. The respective least squares means in first generation were 8.89±0.29 and 8.63±0.19. (Sivaraman et al., 2005) observed wide range of variability in the base population of SDL broiler chickens for response to SRBC with their least squares mean as 6.289±0.246.

(Janeway et al., 2005) Performed that The antibody response is a part of the acquired immune system, which is produced by B cells and mediated by Th2 cells in response to antigenic in young chicks is immature, but a large range of responsiveness has been observed.

(Hatkin et al., 1993) observed the antibody response in young chicks is immature, but a large range of responsiveness has been observed. Chicks generally respond faster and more strongly with increasing age which is probably, among other factors, related to the development of the immune system. (Janeway et al., 2005) Reported that the antibody response was also assumed to be affected by
challenge because of the definition of the antibody response as a response to antigenic stimulation. The magnitude of the antibody response increases with an increasing acute phase response and challenge load but decreases with an increasing maternal immunity.

(Zhang et al., 2006 and Cheema et al., 2007) Observed The bursa of Fabricius is a key lymphoid organ that is responsible for the development and maturation of B-lymphocytes and the humoral antibody response is dependent on this central organ for example, a high antibody response to SRBC has been associated with a larger bursa size in White Leghorn chicken strains.

2.1.2. Serum lysozyme activity

(Biggar and Sturgess, 1977) Reported that Lysozyme plays an important role in body's defense against infection. Its role as an antibacterial agent is mediated through stimulatory effect on macrophage phagocytic function (Thacore and Willet, 1966) as well as its direct bacteriolytic action.

(Di Luzio, 1979) Reported that recently, it has been asserted that serum lysozyme activity reflect the homeostatic expression of the reticulo-endothelial system, which is one of the most fundamental defense mechanism against infection. Moreover, experiments have produced evidence for lysozyme being an index of macrophage functional status. (Hill and Porter, 1974; Jolles and Jolles, 1984)
Observed that the cooperation of lysozyme and antibody/complements in lysis of gram-negative bacteria has been extensively reviewed. (Bessarabov and Krykanov, 1985) observed a positive correlation between dam’s serum lysozyme level and natural resistance of progeny to diseases in chicken.

(Van der Zijpp and Nieuwland, 1986) studied lysozyme level in high and low lines for antibody response to sheep RBCs in ISA Warren chicken. They found that the blood lysozyme level and CMI (in vivo and in vitro) were same in both the lines.

(Pal et al., 1992) while analyzing the genetic divergence among Guinea fowl varieties, assayed serum lysozyme levels in different genetic groups and found significant difference (P<0.01) among the genotypes, but without any definite trend.

(Saxena, 1993) assayed the serum lysozyme by photometric method using *M. lysodeickticus* as substrate in Guinea fowl, desi fowl and commercial broilers; the overall means being 1.18±0.03, 1.79±0.07 and 1.33±0.06 µg/ml, respectively. In guinea fowl, the females had significantly higher values than males. (Pal et al., 1996) estimated serum lysozyme level in guinea fowl at 4 weeks of age and found that serum lysozyme level was 1.01 µg/ml and 0.93 µg/ml in males and females,
respectively. The males showed higher lysozyme activities up to 12 weeks of age.

(Nath et al., 1999) studied the serum lysozyme level in Naked neck (NN) birds and their crosses with normally feathered (nn) birds at the age group of 15-18 weeks. They observed that overall mean for serum lysozyme was 3.24±0.09 µg/ml. Males showed significantly higher level of lysozyme (3.54±0.12 µg/ml) than females (3.18±0.12 µg/ml). The serum lysozyme level in progenies of NN×NN, NN×nn and nn×NN were 3.81±0.19, 3.60±0.12 and 2.67±0.12 µg/ml, respectively and the differences were significant.

(Nath, 1999) observed significant influence of genetic groups on serum lysozyme level in different genetic groups of broiler chicken. (Shivakumar, 2003) assayed the serum lysozyme level using *M. lysodeikcticus* as substrate in IWG and IWJ lines of WLH chickens and observed considerable difference in the values, which were 2.18±0.04 and 1.26±0.04 µg/ml in IWG and IWJ lines respectively.

(Sivaraman, 2004) estimated least squares mean of serum lysozyme level as 1.860±0.047 µg/ml in broiler chicken.

### 2.1.3. Serum Immunoglobulin-G (IgG) level

Humoral immunity is one of the most important body’s defense mechanisms. Humoral immunity is the primary function/effect through
body fluid molecules, particularly the heterogeneous group of immunoglobulins (IgG and IgM) and many other immunologically relevant effector substances. IgG is the most abundant antibody and it constitutes approximately 80% of the total serum immunoglobulins (Kuby, 1997) and is traceable in all body fluids.

In most cases, correlations between anti-SRBC response and serum IgG level has been found to be positive but of low magnitude (Chao and Lee, 2001). By moderating the humoral and cell mediated immune responses, this correlation magnitude can be enhanced many folds (Saxena et al., 1997). Similarly the phenotypic correlation ($r_p$) between HA titre and serum IgG level also been found to be positive but lower in magnitude (Shivraman et al., 2003).

(Chhabra and Goel ,1980) estimated the serum IgG level between 1.0 and 13.5 mg/ml with a mean of 5.09 mg/ml in White Leghorn birds by single radial immunodiffusion (SRID) method; the distribution being nearly normal. (Rees and Nordskog ,1981) analyzed the serum IgG level of ten different inbred chicken lines with a mean ranging from 6.6±0.33 to 13.5±0.68 mg/ml. The heavy breed line W showed the lowest (6.6±0.33 mg/ml) and Leghorn HN showed the highest (13.5±0.68 mg/ml) level.
(Sato et al., 1986) reported that IgG levels in high and low lines for IgG in Plymouth Rock chicken, were 1200-1600 mg/dl and 500-600 mg/dl. The significant differences observed between high and low lines were ascribed to the genetic factor or factors included in selection.

(Ahrestani et al., 1987) estimated the serum IgG levels in different breeds of chicken by Single Radial Immunodiffusion (SRID) method. Aseel (20.51±0.22 mg/ml) had significantly higher level than White Leghorn (7.53±0.61 to 15.99±2.2 mg/ml). (Scott et al., 1988) studied the IgG levels in serum of two lines i.e. large bursal line (LBL) and small bursal line (SBL) of chicken and reported that LBL had higher serum IgG level (10.5±5.07 to 83.44±20.16 mg%) than SBL line (3.8±7.9 and 57.63±12.27 mg%). It was suggested that genes influencing the IgG levels might not be associated with the blood group system.

(Saxena, 1993) studied serum IgG levels in Guinea fowl, Kadaknath and broilers, which averaged 12.19±0.10, 10.01±0.4 and 8.1±0.4 mg/ml, respectively. The high IgG levels were observed at one and two weeks of age (15.7±0.49 and 14.3±0.32 mg/ml), which declined rapidly till fourth week of age and finally attained a static level.

(Sarker et al., 1998) studied the effect of chicken MHC (B complex) on IgM and IgG production, cell mediated immune response and immunity against different disease agents in two pairs of B congenic chicken lines. The MHC type was shown to have significant effect on
serum IgM and IgG production. Heterozygotic advantage of B haplotype on IgM and IgG production was also observed.

(Sarker et al., 1999) studied the direct and correlated response to divergent selection for serum IgM and IgG level in chicken and found that the total antibody titres to SRBC were significantly (P<0.01) higher in low IgG line than in the high IgG line at both 7 and 14 dpi. From the results, it was suggested that selection of chicken on the basis of serum immunoglobin isotypes might change antibody producing cells as well as other immunocompetent cells that modulate the immune response of selected lines.

(Chao-ChingHsein et al., 2000) studied the reproductive and immunological traits in two Taiwan country chicken lines (H and L lines) that had been divergently selected for high and low serum gamma-globulin percentage at 34 weeks of age. The H lines had significantly higher serum gamma-globulin percentage than that of L line. The h² of serum gamma globulin estimated from sire variance component and parent offspring regression was in the range of 0.218 - 0.529. Estimate of realized h² for serum gamma globulin was 0.330. Sivaraman et al., (2001) recorded mean serum IgG level of 7.81±0.17 mg/ml in synthetic dam line of broiler birds.
(Shivakumar, 2003) estimated mean serum IgG concentration in IWG and IWJ genotypes of WLH chickens as 10.66±0.22 mg/ml and 10.52±0.16 mg/ml, respectively. High SRBC response line had significantly higher titres than low SRBC line in both the genotypes.

2.2. DNA polymorphism in IL-2Rγ, IL-15Rα and IFN-γ promoter genes

Candidate gene analysis is a powerful approach to detect variations in the genes controlling traits of economic importance in farm animals, such as immune response (Rothschild and Soller, 1997). Genetically distinct and highly inbred (> 99%) chicken lines, the Leghorn GB.1 and MHC congenic Fayoumi M 5.1 and M 15.2 Lines were used as parental lines to study the polymorphism of Interferon-γ, Interleukin-2 and Immunoglobin Light chain. PCR resulted in 659 bp product of IL-2 gene from Leghorn and Fayoumi lines. Sequencing revealed an A/G mutation at base - 425 between Leghorn and M5.1 (Zhou et al., 2001).

Interleukin 2 (IL-2) is an essential cytokine secreted mainly by activated T lymphocytes and plays a pivotal role in the replication and differentiation of T and B lymphocytes, monocytes, and natural killer cells (Rubin, 1995).

(Farner et al., 1997) Observed the important immunoregulatory role of IL-2, as well as the therapeutic prospects it holds for the
treatment of certain cancers and infectious diseases, has made it the focus of numerous structure–function studies.

(Ohbo et al., 1995) Performed that the interleukin-2 receptor-γ chain (IL-2Rγ) is shared by receptor complexes IL-2, IL-4, IL-7, IL-9 and IL-15, all of which are cytokines involved in lymphocyte development and/or activation. The IL-2Rγ plays a pivotal role in formation of the complete IL-2 receptor, and mutations of the γ-chain gene cause human X-linked severe combined immunodeficiency, resulting in complete or profound T-cell defects.

(Gross et al., 1980) reported that the line selected for high antibody to SRBC exhibited greater antibody response to Newcastle disease and more resistance to Mycoplasma gallisepticum, Eimeria necatrix than the low antibody line, but was more susceptible to Escherichia coli and Staphylococcus aureus.

(Kuby, 1997) Analise that Interferon-γ (IFN-γ) plays a critical role in immune system by increasing the expression of MHC class I and class II molecules that ultimately modulate immune response.

(Zhou et al., 2001). Reported that the promoter region of IFN-γ located upstream from the initiation site plays an important role in regulation of gene expression Correlation between IFN-γ Promoter Polymorphism and resistance to *Escherichia coli* infections in chicken.
Several transcription regulatory elements have been identified in the mammalian IFN-γ gene promoter, such as the TATAATA box, the GATA motif, GM-CSF/MIP motif and consensus octamer site (ATGCAAAAT) (Penix et al., 1993).

(Kaiser et al., 1998) observed that some of these elements are conserved in chickens, such as TATAATA box, GM-CSF/MIP motif and potential NF-κβ family member binding site but the octamer site has not been reported in chickens.

(Zhou et al., 2001) performed that genetically distinct and highly inbred (>99%) chicken lines, the Leghorn GB.1 and MHC congenic Fayoumi M 5.1 and M 15.2 Lines were used as parental lines to study the polymorphism of Interferon-γ, Interleukin-2 and Immunoglobulin Light chain. PCR resulted in 659 bp product of IL-2 gene from Leghorn and Fayoumi lines. Sequencing revealed an A/G mutation at base –425 between Leghorn and M5.1.

(Singh, 2005) observed monomorphic PCR - RFLP patterns in IL-2R_ and IL-15R_ genes with Hph I and Alu I restriction enzymes respectively, in divergent WLH lines. Polymorphic and monomorphic PCR-RFLP patterns in IL-2R_ and IL-15R_ genes with Hph I and Alu I restriction enzymes respectively, in Aseel native chicken observed by (Kumar, 2006).
(Estess et al., 1999; Trentin et al., 1997) Observed that the Interleukin-15 (IL-15) is a pro-inflammatory cytokine that has similar biological functions to IL-2, another cytokine that has previously been identified as a candidate in immune response. Like IL-2, IL-15 is involved in recruiting T cells to tissues during immune response, proliferation of T cells and Natural Killer cells, growth stimulation of B cells, and immunoglobulin synthesis (Kumaki et al., 1996).

(Zhou and Lamont, 2003) reported that Inbred chickens of F$_2$ generation were studied for DNA polymorphism in six candidate genes. IL-2R$_\gamma$ and IL-15R$_\alpha$ were two of them, which showed polymorphism. A 600 bp product of IL-2R$_\gamma$ was sequenced and a T-C SNP between the Leghorn and Fayoumi M 5.1 Lines was observed but no polymorphism was found between Leghorn and Fayoumi M15.2 Lines. For IL-15R$_\alpha$, the amplified 123 bp showed a G-A SNP between the Leghorn and the Fayoumi lines.

(Bulfone-Paus et al., 1999) Reported that Interaction between IL-15 and the IL-15 receptor-$\alpha$ (IL-15R$\alpha$) chain compete with tumor necrosis factor and the tumor necrosis factor receptor-1, a complex that stimulates an apoptotic pathway. (Bulfone- Paus et al., 1999) performed Cytokine IL-15 and its receptor have been implicated in the pathways of several disease processes such as leukemia, cancer, and inflammatory bowel disease. (Singh , 2005) observed monomorphic PCR-RFLP
patterns in IL-2Rγ and IL-15Rα genes with *Hph I* and *Alu I* restriction enzymes respectively, in divergent WLH lines.

(Kumar, 2006) observed polymorphic and monomorphc PCR-RFLP patterns in IL-2Rγ and IL-15Rα genes with *Hph I* and *Alu I* restriction enzymes respectively, in Aseel native chicken. Interleukin-2Rγ (IL-2Rγ) is shared by a receptor complex of many interleukins and it induces proliferation and differentiation of T, B, and NK cells. The chicken B-cell marker (ChB6) gene has been proposed as a candidate gene in regulating B-cell development (Tregskes *et al.*, 1996).

ChB6 alleles are associated with the expression level of MHC class II, regression of Rous sarcoma, and resistance to Marek’s disease (Gilmour *et al.*, 1986; Tregaskes *et al.*, 1996). With regard to the poultry production, the most important poultry production system prevailing in the hill agro ecosystem of the state is rural poultry farming, which is dominated by indigenous chicken resources (Katoch *et al.*, 2010).

(Zhou and Lamont, 2003) reported earlier by PCR–RFLP analysis of IL-2Rγ gene in the present study revealed similar pattern (LL, LF and FF) in Leghorn /Fayoumi chicken. (Jaiswal *et al.*, 2009) observed in Kadaknath native chicken and (Kumar *et al.*, 2007) in Aseel native Chicken. The frequency of heterozygotes (23%) observed in the present study was lower than those reported earlier by (Jaiswal *et al.*, 2009) in Kadaknath native chicken (32%) and (Kumar *et al.*, 2007) in Aseel native...
Chicken (36%) which can be attributed to the differences in the population structure.

(Singh, 2005) observed monomorphic PCR-RFLP patterns in IL-2R_ and IL-15R_ genes with Hph I and Alu I restriction enzymes respectively, in divergent WLH lines. Polymorphic and monomorphic PCR-RFLP patterns in IL-2R_ and IL-15R_ genes with Hph I and Alu I restriction enzymes respectively, in Aseel native chicken observed by (Kumar, 2006).

(Kumar et al., 2007) while analyzing DNA polymorphism in IL-2R_gene with PCR-RFLP in Aseel Indian native chicken, noticed polymorphism; estimated gene frequencies of A and B allele as 0.82 and 0.18 and genotype frequency of AA, AB and BB, analogous to LL, LF and FF reported by (Zhou and Lamont, 2003) as 0.64, 0.36 and 0.0. Monomorphic PCR-RFLP patterns in specialized Layer Chicken Lines of IL-2R_ and observed only 545 bp i.e. BB or the FF genotype observed by (Singh et al., 2008). The wide variations observed in the present study might be due to the reason that no artificial selection has been applied on this breed for immune response or production traits.

Interleukins form an important group of cytokines, which play significant role in eliciting immune responses. IL-2 and IL-15 are pro-inflammatory cytokines that have been identified as candidates in immune response (Trentin et al., 1997; Estess et al., 1999). IL-2
receptor gamma chain (IL-2Ry) is shared by receptor complexes of IL-2, IL-4, IL-7, IL-9 and IL-15, all of which are cytokines involved in lymphocyte development and activation (Zhou et al., 2001). Interactions between IL-15 and the IL-15 receptor alpha (IL-15Ra) chain compete with tumour necrosis factor and the tumour necrosis factor receptor-1, a complex that stimulates an apoptotic pathway (Bulfone-Paus et al., 1999).

The significant role of IL-2Ry and IL-15Ra genes in physiological or regulatory immune function makes them ideal candidates to identify DNA polymorphism. Present investigation was carried out to identify polymorphism at IL-2Ry and IL-15Ra genes with PCR-RFLP and evaluate their association, if any, with humoral immune response to sheep erythrocytes.

2.3. Association between IL-2Rγ, IL-15Rα and IFN-γ promoter genes polymorphism with immunocompetence traits

(Zhou et al., 2001) studied the interferon-γ, Interleukin-2, and IgL gene promoter gene polymorphism and antibody response kinetics in chicken and found that interferon-γ promoter polymorphism had most frequent association with antibody response parameters. There were significant main associations (P<0.05) between interferon-γ promoter polymorphism and antibody response parameters. It was suggested that
interferon-γ gene plays an important role in chicken primary and secondary antibody response to SRBC and there exist interaction among genes for antibody production.

(Zhou et al., 2003) studied the association of six candidate genes with antibody response kinetics in hen and found that most of the significant associations were clustered with IAP-1, Interleukin-15Rα and ZOV3. Interleukin-15Rα polymorphism had most frequent association with antibody response parameters. There were significant main associations (P<0.05) between Interleukin-15Rα polymorphism and antibody response parameters. It was suggested that the SNP in this gene might serve as genetic markers for enhancement of humoral immune response capacity in the chicken.

(Singh, 2005) observed highly positive genetic correlations among three IC-traits viz., haemagglutination (HA), lysozyme (LZM) and immunoglobin-G (IgG) in WL chicken. The r_p among them was also positive but very low in magnitude. (Sivaraman et al., 2003) reported positive (0.06) but significant rp between HA titre and serum lysozyme level but observed a negative (-0.01) and non-significant correlation between HA titre and serum IgG level. The rp between serum lysozyme and IgG was positive (0.04) and non-significant.
(Kumar, 2006) observed genetic correlations among three IC-traitsviz., haemagglutination (HA), lysozyme (Lyso) and immunoglobulin-G (IgG) in Aseel native chicken. (Van der Zijpp, 1983) evaluated humoral immune response of White Plymouth Rock and White Leghorn (WL) chickens to SRBC, but did not observe any correlation between antibody titres to SRBC, on 3, 7, 14 dpi and PHA-P response, neither overall nor within groups of different genotypes. It was suggested that the selection for general immune responsiveness should include parameters of antibody and cell mediated immune responses.

(Cheng and Lamont, 1988) conducted a detailed study for the phenotypic correlations among immunological traits. Where in, the correlation among antibody response, phagocytosis and T-cell mediated immunity were mostly found to be non-significant (0.14-0.17) but the difference in correlation coefficient in males and females were significantly different. Their results indicated the relative independence of genetic control of these components.

(Saxena et al., 1997) observed the phenotypic correlation of anti-SRBC antibodies in Guinea fowl to be 0.22±0.27, 0.62±0.46 and 0.27±0.25 with phagocytic index (PI), IgG and wing index (WI), respectively. The serum IgG concentration had a low $r_p$ with anti-SRBC response (-0.043) in Taiwan country chicken (Chao and Lee, 2001).
(Kumar, 2006) observed two genotype were found in case of IL-2Rγ gene. Heterozygotes at IL-2Rγ gene had higher HA titres than the homozygote observed in this study. They revealed higher titre than the population mean too. However Lysozyme level in heterozygotes was lower than that in both homozygotes and overall population.

Only one genotype was found in case of IL-15Rα gene. Heterozygotes at IL-15Rα gene had much lower HA titres than the population average. Average value for HA, Lysozyme and IgG were 10.7±2.5, 3.8±0.41 and 56.75±9.77, respectively. Serum lysozyme level in heterozygotes was higher than population mean but other two traits were lower than the population mean.

Candidate genes selected for studying immune response traits may have known physiological functions with immune response or be in regulatory or biochemical pathways affecting immune response (Zhou and Lamont, 2003).

After QTL are mapped to a chromosomal region, positional candidate genes can be identified among the genes mapped to that region or by comparative genomic analysis with regions of conserved synteny in other species (Rothschild and Soller, 1997). For the present study, four candidate genes, natural resistance associated macrophage protein 1 (SLC11A1), prosaposin (PSAP), interferon-γ (IFNG), and toll-
like receptor 4 (TLR4), were investigated, based upon prior reports of association with immune response in chickens (Zhou et al., 2001; Kramer et al., 2003; Malek et al., 2004). Association of a SNP polymorphism in a high conserved region of SCL11A1 with *Salmonella enteritidis* (SE) vaccine and pathogen challenge response in young chicken has been reported (Liu et al., 2003).

Interferon-γ (*IFNG*) is a pleiotropic cytokine involved in most phases of immune and inflammatory responses, and has been shown to prime heterophil functional activities (Kogut et al., 2005).

Associations between *IFNG* promoter polymorphisms and chicken primary and secondary antibody response to SRBC and *Brucella abortus* (BA) have been reported (Zhou et al., 2001). A recent report (Ye et al., 2006) demonstrated associations of an *IFNG* promoter SNP with infectious bursal disease virus vaccine response in broilers.

In the (Ye et al., 2006) study, the same *IFNG* SNP allele that was associated with higher BW and lower mortality, was associated with lower antibody response to infectious bursal disease vaccine. Cumulatively, these studies and others (Siegel and Gross, 1980; Martin et al., 1990; Mashaly et al., 2000) indicate a negative association between the effect of gene polymorphisms on BW and their effects on antibody response parameters, perhaps due to the elevation in metabolic rate of the high immune response genotype.
Toll-like receptors (TLRs) are a family of genes whose proteins are the main sensors utilized by the innate immune system to detect invading pathogens. (Werling and Thomas, 2003) showed that TLR are crucial not only in the early phase of infection when innate immunity is important, but also link innate and adaptive immunity through the entire course of the host defense response. The TLR4 in the chicken has been associated with response to SE (Leveque et al., 2003). (Dil and Qureshi, 2002) demonstrated that TLR4 expression is required for production of lipopolysaccharide-mediated inducible nitric oxide in chicken macrophages.

The relative number of TLR4 receptor molecules on the macrophage surface varies between chicken strains, thereby varying the level of LPS binding among strains (Dil and Qureshi, 2002). The goal of the current study was to identify associations of SNP in four genes with antibody response kinetics and body weight in chickens.

To explain the genetic control of growth of rapidly growing chickens, apoVLDL-II gene may be used as candidate gene for growth and body composition traits. A few studies have examined association between single nucleotide polymorphism of apoVLDL-II gene with growth and body composition traits in the chicken (Li et al., 2005; Musa et al., 2007). Therefore, the present study was developed to detect apoVLDL-II gene polymorphism in Iranian commercial broiler lines and
evaluate the associations between apoVLDL-II SNP with growth and body composition traits based on PCR-RFLP methods.

(Li et al., 2005) founded a G/A mutation at base 634 (GenBank accession no: V00448) in the first intron of the apoVLDLII gene. Although this mutation was not located at an identified protein-binding site, the polymorphism was associated with growth, gain and skeleton and body composition traits of growing birds. Therefore, SNP in the first intron of the apoVLDLII gene might, have been closely linked with functional polymorphism in other regions of the apoVLDLII gene or the other linked genes.

Growth is under complex genetic control and considering the molecular mechanisms of growth will improve selection strategies in broiler chickens. In the present study, the apoVLDL-II SNP had an association (P < 0.05) with BW6 (g), CW, BMW and DW. This result was similar to results of (Li et al., 2005 and Musa et al., 2007). In these populations, birds with AB genotype were significantly heavier than the BB genotypes (P ≤ 0.05) for BW6 (g), CW, BMW, DW and WINW traits. The allele association is consistent with the selection history of broilers emphasized on increasing of Market age. In this study, apo VLDL-II SNP was not significantly (P > 0.05) associated with BAKW and AFW. This was similar to results of (Li et al., 2005). In contrast, (Musa et al., 2007)
reported that the studied SNP in apo VLDL-II gene significantly (P < 0.05) affect AFW.
3-AIM AND OBJECTIVES

The poultry industry in India has extensively grown in the past three decades. However, there is still a possibility of further growth in this sector. Such a growth in poultry production can be achieved by adopting better breeding management programmes that involve producing healthy, disease-resistant breeds of chicken.

Aseel has plenty delicious flavoured meat due to its biggest body size among the Indian native breeds of chickens accompanied by broader breast. The male birds grew faster than female birds and attained the maximum weight gain at 15\textsuperscript{th} week of age. Seven non linear functions were fitted to average weekly body weights of male and female birds.

For this, identification of better breeds of fowl that have high immunocompetence is quite critical. Therefore, this study aims to assess the immunocompetence status of native breed of chickens and to further identify the gene polymorphism in Interleukin gene(s), namely IL-2R\textsubscript{γ}, IL-15R\textalpha{} and IFN-γ, by PCR-RFLP. Moreover, an association between DNA polymorphism in these genes and the immunocompetence traits of the birds is also determined. A meta-analysis of experiments increasing/decreasing clutch/brood size to alter parental effort found a significant negative relationship between parental effort and immune
responses. A study was conducted to determine the variation in physical character and Immunocompetance among native breed of chicken.

Three candidate genes, interferon-gamma (IFN-gamma), interleukin-2 (IL-2), and immunoglobulin G light chain (IgL) were studied. Primers for the promoter regions were designed from EMBL chicken genomic sequences. Polymorphisms between parental lines were detected by direct sequencing. Heterozygotes at IL-15α gene however, revealed significantly lower HA titer than the population average. Aseel breed of native chicken showed higher immunocompetence status than that reported in other chicken breeds.

- To assess Immunocompetence status of native breed of chickens.
- To identify the candidate gene polymorphism in Interleukin gene(s) by PCR-RFLP.
- To determine the association among DNA polymorphism and Immunocompetence traits.
4-MATERIALS & METHODS

4.1. EXPERIMENTAL ANIMALS

4.1.1. Aseel Birds

In the present investigation, 251 random bred birds belonging to Aseel breed of indigenous chicken, being maintained at Desi foul unit of CARI, Izatnagar were used.

4.1.2. Sheep

Healthy Muzaffarnagari breed of sheep maintained at Sheep and Goat farm of Livestock Production Research Centre, Indian Veterinary Research Institute, Izatnagar were used for collection of blood, which was used in humoral immune response studies.

4.2. Evaluation of immunocompetence traits

The immunocompetence status of the birds can be assessed by analyzing various components of immune system. A few important facets of immune responses which contribute to the immunocompetence of the birds, viz. humoral response manifested by its components like antibody titres against Sheep red blood cells (SRBC), serum IgG level and serum Lysozyme activity were evaluated in this investigation.
4.2.1. Humoral immune response to sheep RBC

The immune response to SRBC was assessed through HA test as per (Van der Zijpp and Leenstra, 1980).

4.2.1.1. Preparation of sheep RBC antigen

20 ml blood was collected from jugular vein of healthy sheep in a sterile heparinized (20 IU/ml) test tube. It was centrifuged at 1500 rpm for 10 minutes at 4°C to settle down the RBCs. The RBCs were then washed thrice with PBS, pH 7.2 (refer Annexure for composition) by mixing and centrifuging it to remove other serum components. Finally, 1% (V/V) sheep RBC suspension was prepared by mixing 1 ml of packed sheep RBCs and 99 ml of PBS, which was then used for injection in the birds as antigen.

4.2.1.2. Immunization of birds with SRBC

One ml of 1% sheep RBC suspension was injected into the jugular vein of each bird with tuberculin syringe. Jugular vein was the choice of injection as it led to minimum bleeding in comparison to other veins like brachial vein etc.

4.2.1.3. Harvesting of immune sera from SRBC sensitized birds

2 ml blood was collected in sterilized glass tubes on 5th day post immunization (5 dpi) and allowed to clot for 2-3 hours at 37°C. The hyper
immune sera oozed out of the clot. Sera samples were collected in 0.5 ml sterile tubes. Samples in which the volume of serum was insufficient, clot was broken gently and tubes were centrifuged at 1500 rpm for 3-4 min. to collect serum. Sera samples were stored at -20°C till further analysis.

4.2.1.4. Estimation of antibody titer against sheep RBCs

The antibody response to SRBC was assessed using haemagglutination test (Van der Zijpp and Leenstra, 1980) as mentioned below: -

- The test was performed in round bottom (U shaped) microtitre plates. 50 µl of phosphate buffered saline (PBS) was added in each well. Then, 50 µl of serum was added in first well of each row except the last row where 50 µl of PBS was added, it acted as control.

- After through mixing, the sera were two fold serially diluted by taking 50 µl from first wells of each row and transferring them to subsequent wells. This process was continued till last column from where 50 µl was discarded.
Equal volume (50 μl) of 1% SRBC suspension was then added in all the wells followed by thorough mixing by rocking on table surface. The plates were then incubated at 37°C for 1 hour in a humid chamber. The highest dilution (n), which yielded complete agglutination (button shaped clumping of RBCs indicated haemagglutination reaction), was recorded as titer and expressed as log₂ n.

4.2.2. Estimation of serum Lysozyme level

The serum lysozyme concentration was estimated using Lysoplate assay method (Lie et al., 1986).

4.2.2.1. Reagents and chemicals required

A. Dibasic buffer 0.066 M, pH 6.3 (Refer Annexure for composition).

B. Micrococcus lysodiekticus

C. Standard Lysozyme

D. Agarose

4.2.2.2. Methodology

The lysozyme standards were prepared by dissolving 2 mg of standard lysozyme (SRL, India) in 1 ml of dibasic buffer. Various dilutions were prepared so as to bring the final concentration of...
lysozyme as 40 μg/ml, 20μg/ml, 10μg/ml, 5.0 μg/ml, 2.5 μg/ml and 1.25 μg/ml by serial dilution.

- The agar lysoplate was set up on a perfect horizontal surface. The glass plate was cleaned and sterilized with spirit and air-dried. The borders were prepared placing glass strips on the edges of required area. All the four sides of the borders were sealed with 2% agar.

- Volume for 0.5 cm thick gel was calculated by formula:

  \[
  \text{Volume} = \text{Length} \times \text{Width} \times 0.5
  \]

- 50 ml of 1% Agarose in dibasic buffer was sufficient for 15 X 15 cm\(^2\) plate. After boiling the Agarose in dibasic buffer it was cooled to 60°C and the prediluted *Micrococcus lysodeiketicus* (Sigma, USA), 50 μg per ml of dibasic buffer, was added into it and mixed well.

- Then the whole content was poured onto the bordered and sealed glass plate, spread uniformly and was left at room temperature for polymerization. After polymerization of gel, the wells were punched at a distance of 1.5 cm with the help of a gel punch. 10 μl of serum sample was added to each well. Lysozyme standard samples were
also loaded in the wells at one side (4-5 dilutions). The plate was incubated at 37°C in humidity-controlled chamber for 24 hr.

- It was stained with 0.2% Coomassine Brilliant Blue for 6 hours and excess stain was removed with destaining solution. The diameters of the lysed zones were measured with Digital Vernier calipers.

4.2.2.3. Determination of Lysozyme concentration

The concentrations (After log$_2$ transformation) of standards were regressed on diameter of the lysed zones around these standards. The slope of the curve and intercept were determined. The lysozyme concentration in the unknown sera samples were determined by following regression equation:

\[ Y = bx + c \]

Where,

\( Y = \) Concentration of unknown sample  
\( b = \) Slope of regression equation  
\( c = \) Intercept of regression equation  
\( x = \) Diameter of the lysed zone around unknown sample.
4.2.3. Estimation of serum IgG by Single Radial Immunodiffusion (SRID) Assay

Serum IgG is the most abundant antibody and constitutes approximately 80% of the total immunoglobulins. The bird’s ability to mount antibody responses to other antigens is primarily revealed by serum IgG concentration.

4.2.4. Statistical analysis

The data generated on immunological traits were analyzed by LS ANOVA using following Statistical model: -

\[ Y_{ijk} = \mu + S_{xi} + A_j + e_{ijk} \]

Where, \( Y_{ijk} \) = value of a trait measured on \( ijk^{th} \) individual

\( \mu \) = Overall mean

\( S_{xi} \) = Effect of \( i^{th} \) sex (\( i = 1, 2 \))

\( A_j \) = Effect of \( j^{th} \) age group (\( j = 1, 2, 3, 4 \))

\( e_{ijklm} \) = random error associated with mean ‘0’ and variance \( \sigma^2 \)
4.3. DNA Polymorphism at IL-2Rγ, IL-15Rα and IFN-γ promoter genes by PCR-RFLP

Nucleotide polymorphism at two interleukine receptor genes i.e. interleukin 2 receptor-γ, interleukin-15 receptor-α (IL-15Rα) and one promoter gene, interferon gamma (IFN-γ) were studied by PCR-RFLP.

4.3.1. Genomic DNA isolation

Sixty birds (20 each at high, middle and low extremes) were selected after screening of 251 birds for their response (HA titres) to SRBCs. Genomic DNA samples were isolated from these birds using Phenol extraction method (Kagami et al., 1990). Brief methodology is presented below-

- 0.1 ml of blood was collected from jugular vein in heparinized tube. It was subjected to centrifuge at 3000 rpm for 5 min with PBS in refrigerated centrifuge for washing of cells.
- The supernatant was discarded and ml of lysis buffer was added. RBCs were suspended in the lysis buffer and kept at room temperature for 15-20 min. Then, proteinase K at a final concentration of 200µg/ml and SDS at final concentration of 200µg/ml 0.5% were added and it was kept overnight in water bath
at 37°C for digestion. In this mixture equal volume of Tris-saturated Phenol was added and mixed gently for 5 minutes.

- Two phases were separated by centrifuging at 5000 rpm for 5 min and the upper aqueous phase was transferred to a new microfuge tube and this step was repeated twice. The aqueous phase was then extracted twice with equal volume of Phenol: Chloroform: Isoamyl Alcohol (25:24:1).

- Lastly, the aqueous phase was extracted twice with equal volume of Chloroform: Isoamyl Alcohol (24:1). DNA was precipitated from the aqueous phase by adding 2-2.5 volumes of chilled absolute ethanol gentle mixing. It was kept at -20°C till further use.

- The precipitated DNA was centrifuged at 10000 rpm for 10 min and the DNA pellet was washed twice with 70% ethanol. Pellet was then air dried and dissolved in sufficient volume (easily pipettable, not so viscous or watery) of autoclaved distilled water.

4.3.2. Estimation of DNA concentration and purity checking

The concentration of DNA was calculated spectrophotometrically by taking optical density (OD) at 260 nm using the following formula:

\[
\text{DNA concentration (µg/ml)} = \text{OD}_{260} \times \text{Dilution factor} \times 50
\]
The purity of DNA was assessed by calculating ratio of optical densities at 260 and 280 nm. The samples with OD ratio (260nm/280nm) ranging from 1.7 to 1.9 were used in subsequent experiments.

4.3.3. Assessment of DNA quality

Quality of DNA was assessed through 0.7% horizontal submarine agarose gel electrophoresis as below-

- The gel casting plate was sealed with adhesive tape and placed on a leveled table surface.
- Agarose (0.7% w/v) was boiled in 1X TBE (Refer Annexure for composition) buffer. After boiling it was cooled to 55°C and then ethidium bromide (0.5 µg/ml) was added. The gel was gently poured into the casting tray avoiding bubble formation and was allowed to solidify at room temperature.
- After solidification, the comb and adhesive tape were removed. The gel casting tray was submerged in gel tank of electrophoresis unit having 1 X TBE buffer.
- DNA samples were prepared by mixing 3µl of genomic DNA, 7µl of distilled water and 2µl of Bromo Phenol Blue loading dye. Samples were carefully loaded in the wells.
Electrophoresis was performed at 2-5 volts/cm for one hour and then gel was visualized and photographed under UV transilluminator.

The genomic DNA samples having good quality (Intact bands without smearing) were used for further analysis.

4.3.4. PCR-RFLP Analysis

PCR-RFLP is a simple and reliable technique for studying nucleotide polymorphism in nucleotide sequences in genes. It involves designing of a set of primers for the locus of interest which are used for PCR amplification of that segment of DNA, followed by restriction enzyme digestion of the PCR product and visualization of restriction fragments in gel.

4.3.4.1. Primer preparation

Following primer sequences were got synthesized utilizing the published information and used in this study. The forward and reverse primer for IFN-γ promoter were synthesized according to (Zhou et al., 2001) and IL-2Rγ and IL-15Rα according to (Zhou and Lamont, 2003).
<table>
<thead>
<tr>
<th>Name of genes</th>
<th>Name of Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2Rγ</td>
<td>Upstream</td>
<td>5’ CCC AGC GTG GAC TAT GAG AA 3’</td>
</tr>
<tr>
<td></td>
<td>Downstream</td>
<td>5’ CAT CTT TAG GAC TCC GAC CCA 3’</td>
</tr>
<tr>
<td>IL-15Rα</td>
<td>Upstream</td>
<td>5’ CCT TGG TAG TTC TAG GGC T 3’</td>
</tr>
<tr>
<td></td>
<td>Downstream</td>
<td>5’ CTG GCT GTT GTG TAG GAT T 3’</td>
</tr>
<tr>
<td>IFN-γ promoter</td>
<td>Upstream</td>
<td>5’ GT AAG GAA CTT CAG CCA TTG 3’</td>
</tr>
<tr>
<td></td>
<td>Downstream</td>
<td>5’ GAC GAA TGA ACT TCA TCT GCC 3’</td>
</tr>
</tbody>
</table>

The upstream and downstream primers used to amplify IFN-γ were expected to yield a 670 bp product. Similarly, other primers set used to amplify IL-2Rγ and IL-15Rα were expected to yield 600 and 123 bp products respectively.

### 4.3.4.2. PCR reaction mixture

The PCR was carried out in a total reaction volume of 25μl. The components were optimized using variable concentrations of different components. The optimized reaction that was finally used for amplification was as below:
Table 1. PCR reaction mixture for IL-2Rγ and IL-15Rα

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Reaction component</th>
<th>Volume</th>
<th>Final conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Template (Genomic DNA)</td>
<td>0.5 µl</td>
<td>25 ng</td>
</tr>
<tr>
<td>2.</td>
<td>Up stream primer</td>
<td>1 µl</td>
<td>0.8 µM</td>
</tr>
<tr>
<td>3.</td>
<td>Down stream primer</td>
<td>1 µl</td>
<td>0.8 µM</td>
</tr>
<tr>
<td>4.</td>
<td>10 X PCR buffer</td>
<td>2.5 µl</td>
<td>1 X</td>
</tr>
<tr>
<td>5.</td>
<td>dNTP mix</td>
<td>2 µl</td>
<td>0.2 mM</td>
</tr>
<tr>
<td>6.</td>
<td>Autoclaved triple glass distilled water</td>
<td>17.7</td>
<td></td>
</tr>
<tr>
<td>7.</td>
<td>Taq DNA polymerase 5U/µl</td>
<td>0.3 µl</td>
<td>1.5U</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>25 µl</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. PCR reaction mixture for IFN-γ promoter

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Reaction component</th>
<th>Volume</th>
<th>Final conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Template (Genomic DNA)</td>
<td>2.0 µl</td>
<td>100 ng</td>
</tr>
<tr>
<td>2.</td>
<td>Up stream primer</td>
<td>1 µl</td>
<td>0.8 µM</td>
</tr>
<tr>
<td>3.</td>
<td>Down stream primer</td>
<td>1 µl</td>
<td>0.8 µM</td>
</tr>
<tr>
<td>4.</td>
<td>10 X PCR buffer</td>
<td>2.5 µl</td>
<td>1 X</td>
</tr>
<tr>
<td>5.</td>
<td>dNTP mix</td>
<td>2 µl</td>
<td>0.2 mM</td>
</tr>
<tr>
<td>6.</td>
<td>Autoclaved triple glass distilled water</td>
<td>16.2</td>
<td></td>
</tr>
<tr>
<td>7.</td>
<td>Taq DNA polymerase 5U/µl</td>
<td>0.3 µl</td>
<td>1.5U</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>25 µl</td>
<td></td>
</tr>
</tbody>
</table>

4.3.4.3. PCR amplification Program

PCR amplification was carried out in a thermal cycler (PTC-200, MJ Research, and USA) as per (Zhou et al., 2001) and (Zhou and Lamont, 2003) using following cyclic conditions.
<table>
<thead>
<tr>
<th>IL-2Rγ</th>
<th>IL-15Rα</th>
<th>IFN-γpromoter</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Initial denaturation at 94°C for 5 min.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. 35 cycles of</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a). Denaturation at 94°C for 1 min.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>b). Annealing at 53.7°C for 1 min.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>c). Extension at 72°C for 1 min.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. Final extension at 72°C for 15 min.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Initial denaturation at 94°C for 5 min.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. 35 cycles of</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a). Denaturation at 94°C for 1 min.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>b). Annealing at 55°C for 1 min.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>c). Extension at 72°C for 1 min.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. Final extension at 72°C for 15 min.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Initial denaturation at 94°C for 5 min.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. 30 cycles of</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a). Denaturation at 94°C for 1 min.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>b). Annealing at 58°C for 1 min.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>c). Extension at 72°C for 1 min.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. Final extension at 72°C for 15 min.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

4.3.4.4. Documentation of PCR products by Agarose Gel electrophoresis

8 μl of PCR product was added with 2 μl distilled water and 2 μl bromophenol blue dye for loading in a 1.4% agarose gel containing ethidium bromide (0.5 µg/ml). The electrophoresis was done at 2-5 V/cm. 100 bp DNA ladder (Bangalore Genei, India) was used as molecular size marker for identification of the desired product. The amplified product was examined under UV illumination and photographed for documentation.

4.3.4.5. Restriction enzyme digestion

To identify the restriction fragment length polymorphism, one restriction enzyme was used for each amplified product. The RE
digestion was carried out in 30µl as per the manufacturer’s instruction. RE Alu I, Hph I and Tsp 509 I were employed for IL-15Rα, IL-2Rγ and IFN-γ promoter respectively.

**Table.3. Alu1 RE digestion of 123 bp of IL-15Rα gene**

<table>
<thead>
<tr>
<th>Reaction components</th>
<th>Amount (1x)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alu I (20U/µl)</td>
<td>0.2µl</td>
</tr>
<tr>
<td>10x buffer B</td>
<td>3µl</td>
</tr>
<tr>
<td>Autoclaved triple distilled water</td>
<td>11.8µl</td>
</tr>
<tr>
<td>PCR product</td>
<td>15µl</td>
</tr>
</tbody>
</table>

The Alu1 digestion was carried out for 24 hours at 37°C in a water bath. After digestion the digested products were kept in refrigerator at 4°C till further study.

**Table.4. Hph1 RE digestion of 600 bp of IL-2Rγ gene**

<table>
<thead>
<tr>
<th>Reaction components</th>
<th>Amount (1x)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hph I (20U/µl)</td>
<td>0.2µl</td>
</tr>
<tr>
<td>10x buffer R</td>
<td>3µl</td>
</tr>
<tr>
<td>Autoclaved triple distilled water</td>
<td>11.8µl</td>
</tr>
<tr>
<td>PCR product</td>
<td>15µl</td>
</tr>
</tbody>
</table>

The Hph1 digestion was carried out for 24 hours at 65°C in a water bath. After digestion, the digested products were kept in refrigerator at 4°C till further study.

**Table.5. Tsp509 I RE digestion of 670 bp of IFN-γ promoter gene**

<table>
<thead>
<tr>
<th>Reaction components</th>
<th>Amount (1x)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hph I (20U/µl)</td>
<td>0.2µl</td>
</tr>
<tr>
<td>10x buffer R</td>
<td>3µl</td>
</tr>
<tr>
<td>Autoclaved triple distilled water</td>
<td>11.8µl</td>
</tr>
<tr>
<td>---------------------------------</td>
<td>-------</td>
</tr>
<tr>
<td>PCR product</td>
<td>15µl</td>
</tr>
</tbody>
</table>

The *Tsp509 I* digestion was carried out for 24 hours at 65°C in a water bath. After digestion, the digested products were kept in refrigerator at 4°C till further study.

4.3.4.6. Determination of molecular sizes of digests and recording of RFLP pattern

The molecular sizes of the digests were determined with the help of computer program (Schaffer and Sederoff, 1981).

4.3.4.7. Statistical analysis

On the basis of molecular sizes of digests, genotypes were grouped according to reports of (Zhou and Lamont, 2003) with slight changes in terminologies. Standard statistical method was followed to calculate gene and genotypic frequencies.

4.4. Association between IL-2Rγ, IL-15Rα and IFN-γ promoter genes polymorphism with immunocompetence traits

Association between immunocompetence traits and genotypes was calculated by one way ANOVA.
5. RESULTS

5.1. EVALUATION OF IMMUNOLOGICAL TRAITS

The immunocompetence status can be assessed by analyzing various components of immune system viz. antibody titres against SRBC, serum IgG level and Lysozyme activity. Antibody titres against SRBC and serum IgG level act as indicators for humoral immune response where as bacteriolytic activity of serum Lysozyme acts as indicator for non-specific immune response.

5.1.1. Antibody response to Sheep RBC

The antibody titres against sheep RBCs were measured through HA test on 5\textsuperscript{th} dpi. HA titres ranged from 2-21 (Fig. 2). Average HA titre was 12.80±0.74 and 11.96±0.64 in female and males, respectively. The overall average was 12.38±0.60.

Table 6. Least Squares Analysis Of Variance of Various Immunocompetence Traits in Aseel Native Chickens

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>df</th>
<th>HA titre</th>
<th>Serum Lysozyme</th>
<th>Serum IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>1</td>
<td>25.47</td>
<td>0.054</td>
<td>2588.1</td>
</tr>
<tr>
<td>Age group</td>
<td>3</td>
<td>61.66*</td>
<td>1.554</td>
<td>2655.5</td>
</tr>
<tr>
<td>Remainder</td>
<td>246</td>
<td>17.62</td>
<td>1.745</td>
<td>2144.7</td>
</tr>
</tbody>
</table>

*P < 0.05(samples have been taken four replicates.)
Table 7. Least Squares Means±Standard Error of Various Immunocompetence Traits in Aseel Native Chickens

<table>
<thead>
<tr>
<th>Factor</th>
<th>Number of observations</th>
<th>HA titre (μg/ml)</th>
<th>Serum Lysozyme (μg/ml)</th>
<th>Serum IgG (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall</td>
<td>251</td>
<td>12.38±0.60</td>
<td>3.42±0.19</td>
<td>69.87±6.57</td>
</tr>
<tr>
<td>Male</td>
<td>50</td>
<td>12.80±0.74</td>
<td>3.40±0.23</td>
<td>74.09±8.13</td>
</tr>
<tr>
<td>Female</td>
<td>201</td>
<td>11.96±0.64</td>
<td>3.44±0.20</td>
<td>65.64±7.05</td>
</tr>
<tr>
<td>Age group</td>
<td>1</td>
<td>66</td>
<td>10.88±0.54b</td>
<td>3.08±0.17</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>157</td>
<td>11.5±0.40b</td>
<td>3.31±0.13</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>24</td>
<td>14.04±0.92a</td>
<td>3.39±0.29</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>4</td>
<td>13.08±2.13a</td>
<td>3.90±0.67</td>
</tr>
</tbody>
</table>

Means with dissimilar superscripts in a column under a class differ significantly (P<0.05). Samples have been taken four replicates.

Aseel breed of native chicken demonstrated higher HA titre than most of the other breeds. The wide variations observed in the present study might be due to the reason that no artificial selection has been applied on this breed of chicken for immune response or production traits. Survival of the fittest i.e. natural selection has maintained only the immunocompetent population of birds.

In this study effect of sex and age on humoral response to sheep erythrocytes measured as HA titre was studied.

5.1.1.1. Effect of Age

The influence of age was significant (P≤0.05) on HA titre (Table 6.), older birds had higher mean antibody titres (13.0±2.13) than younger birds (10.88±0.54).
5.1.1.2. Effect of Sex

The influence of sex on HA titre was statistically non-significant (Table .6.) although males had higher mean antibody titre (12.80±0.74) than females (11.96±0.64).

5.1.2. Serum Lysozyme Concentration

The serum lysozyme concentration was estimated using Lysoplate assay method. The least squares ANOVA of serum lysozyme level has been given in Table No. 6. And its least square means factor are given in Table. No.7. Serum lysozyme level ranged from 1.23-10.34 μg/ml (Fig. 3). Average serum lysozyme level was 3.40±0.23 and 3.44±0.20 in male and females, respectively. The overall average was 3.42±0.19.

5.1.2.1. Age effect

Age of the birds had no significant (P>0.05) effect on serum lysozyme level. However, older birds revealed somewhat higher values.

5.1.2.2. Sex

The influence of sex on serum lysozyme was non-significant (Sivaraman et al., 2005) also reported that sex had non-significant effect on serum lysozyme.
Present findings suggested that the genetic mechanisms responsible for mounting of antibody responses to sheep RBC and regulation of serum lysozyme levels in Aseel chicken might be independent.

5.1.3. Serum IgG concentrations

Chicken serum IgG neutralizes the anti chicken IgG. Agarose gel was used as a solidifying base to assay IgG concentrations through Single Radial Immuno diffusion (SRID) assay (Manicini et al., 1965) as per following procedure:

- Clean and sterilized glass plate was placed on leveled horizontal surface. The borders prepared with glass strips were sealed with agar.

- 50 ml of 0.1 M Tris- HCl was divided equally into two halves. To the first half 0.75 g Agarose was added @ 3% (w/v) and boiled to dissolve agarose. To the second half 1.750 ml of anti-chicken IgG (Sigma, USA) was added and after thorough mixing, it was kept at 50°C in a water bath.

- The temperature of first half was brought down to 50°C and second half was mixed. The whole content was poured on to the
glass plate. The gel was allowed to solidify for 1-2 hr. Then, wells were punched at a distance of 1.5 cm with the help of gel punch.

- The standards of IgG (Sigma, USA) viz. 20 μg/ml, 10 μg/ml, 5.0 μg/ml, 2.5 μg/ml and 1.25 μg/ml, prepared by serial dilution of stock solution were loaded in the wells to plot standard curve. 5 μl of unknown sera were diluted to 4 times with 0.1 M Tris and then 10 μl of each sample was loaded in the wells. The plate was incubated at 37°C for 24 hr in humid chamber.

- The diameters of the ring around standard as well as unknown samples were measured with the help of Digital Vernier Calipers. The serum IgG concentrations in unknown samples were determined with the help of regression equation obtained by plotting log₂ concentrations of IgG standards against diameter of the precipitation ring as mentioned in serum lysozyme.

The average serum IgG concentration was 69.87±6.57 (Fig. 4.4). It was higher in males (74.09±8.13) than females (65.33±10.20).

5.1.3.1. Age effect

Age of the birds had no significant (P>0.05) effect on serum IgG level. However, older birds revealed somewhat higher values.
5.1.3.2. Sex

Sex of the birds had no significant (P>0.05) effect on serum IgG level. However, male birds revealed higher values.

5.2. DNA Polymorphism in IL-2Rγ, IL-15Rα and IFN-γ promoter genes

DNA polymorphism IL-2Rγ, IL-15Rα and IFN-γ promoter were studied using PCR-RFLP. RFLP patterns were analyzed to study the association of genotypes with immunocompetence traits.

5.2.1. DNA isolation and its evaluation for quality, purity and concentration

Sample amount of genomic DNA were isolated from 60 blood samples (20 each at high, middle and low extremes of HA titres) using Phenol extraction method.

The quality of genomic DNA was assessed by horizontal Agarose gel electrophoresis and found to be good. All samples gave intact bands without smearing on 0.7% Agarose (Fig. No.5). Gel was visualized under UV transilluminator.

Purity and concentration of genomic DNA was determined by optical density (OD) determination at 260 nm and 280 nm in a UV Spectrophotometer. Ratio of $\text{OD}_{260}$ to $\text{OD}_{280}$ of most of the DNA samples
were lying in the range of 1.7 to 1.9 indicating satisfactory purity of DNA samples.

Concentrations of DNA ranged from 321.2- 983.7 ng/µl. DNA samples were diluted with triple glass distilled water to obtain a final concentration of approximately 50 ng/µl. These diluted DNA samples were used in PCR reactions.

![Fig. 5. Quality assessment of genomic DNA of Aseel native chickens on 0.7% Agarose gel](image)

**5.2.2. PCR optimization**

The reaction mixture and PCR programmes for IL-15Rα, IL-2Rγ and IFN-γ promoter genes were optimized as per (Zhou and Lamont, 2001) and (Zhou et al., 2003), respectively with slight modifications.
5.2.3. Optimized PCR reaction mixture

For IL-15Rα and IL-2Rγ the optimum combination of various reaction components for each 25 µl PCR reaction mix was 25 ng genomic DNA, 0.8 mM of each primer 0.2 mM each dNTPs and 1 U Taq DNA polymerase. In case of IFN-γ promoter gene instead of 25 ng, use of 100 ng genomic DNA gave better amplification.

5.2.4. Optimization of PCR programme

To optimize the PCR conditions different annealing temperatures, initial denaturation time and number of cycles were used. In case of IL-15Rα and IL-2Rγ, Initial denaturation at 94°C for 5 min, 35 cycles of (a) Denaturation at 94°C for 1 min., (b) Annealing at 55°C (53.7°C in case of IL-2Rγ) for 1 min., (c) Extension at 72°C for 1 min and final extension at 72°C for 15 min. produced distinct and robust amplified product.

In case of IFN-γ promoter gene, Initial denaturation at 94°C for 5 min, 35 cycles of (a) Denaturation at 94°C for 1 min., (b) Annealing at 58°C for 1 min., (c) Extension at 72°C for 1 min. and final extension at 72°C for 15 min. produced distinct product.

The optimized PCR reaction mixture and PCR programmes gave satisfactory amplification of all the genes in Aseel breed of native chicken.
5.2.5. Polyacrylamide Gel Electrophoresis (PAGE)

10% Polyacrylamide gel was prepared for documentation of RE digested products. The composition of polyacrylamide gel used in the present study is given below.

Reagents for 24 ml

(30%) Acrylamide and N, N'methelene bisacrylamide (29:1) 8.0 ml

5X TBE 4.8 ml

Autoclave triple distilled water 11.2 ml

10% Ammonium per sulphate 200 µl

TEMED 10 µl

5.2.5.1. Procedure

- Glass plates (16×15.5cm) were washed with detergent to make them stain free, rinsed initially under running tap water properly and finally in triple distilled water before drying. After drying, glass plates were cleaned with cotton soaked in ethanol and then dried.

- Glass plates were clamped by putting 2mm spacer in its position after proper sealing. The gel solution was poured gently into the space avoiding bubble formation and comb was inserted
immediately before beginning of polymerization. The gel was allowed to polymerize at room temperature for 45 minute.

- Then comb was removed slowly and wells were washed with autoclaved triple distilled water. After this plate was put in vertical electrophoresis tank with notch plate facing towards the buffer reservoir loaded with 1X TBE, which was leveled properly after putting the plates. Samples were mixed with 6X loading dye and poured in the well with gel loading tip.

- Electrophoresis was done at 12 ampere for 5 min. Initially followed by constant current of 10 ampere for 3-4 hours. The size of the bands separated was calculated with the help of standard DNA molecular markers, which were run along with the digests.

5.2.5.2. Silver staining of PAGE

Silver staining was done according to Sambrook and Russel (2001) with slight modifications as follows:

- The gel was placed on a cleaned glass tray by keeping notch plate facing upwards. Then upper plate was detached gently with the help of a broad surfaced spatula with due care to avoid breakage of gel plate. The first row was marked by cutting upper portion of the gel at one end.
- The gel was flushed with 200 ml of 10% ethanol in a glass tray and DNA bands were fixed with the same solution for 5 minutes. The gel was pretreated (oxidized) with 500 ml of 1.5% nitric acid for 3 min by gentle shaking. The gel was washed properly with triple distilled water for 30 seconds.

- The gel was impregnated with 300 ml of 0.2% AgNO₃ solution for 20 min by shaking gently. The gel was washed properly with triple distilled water for 20 seconds.

- The gel was developed by applying, initially 100 ml of cold developing solution (15 g in 500 ml Na₂CO₃; 0.27 ml of 37% formaldehyde per 500 ml; formaldehyde was added to Na₂CO₃ 15 min. before addition of developing solution) and gently shaken for 30 seconds. Then solution was replaced with another 100 ml of fresh cold solution, and shaken for 1-2 min. This process was repeated until the bands appeared with desirable intensity.

- The developing reaction was stopped by adding 10% glacial acetic acid for 5 minutes. The gel was stored in triple distilled water.
5.2.6. DNA Polymorphism at IL-2Rγ gene

For IL-2Rγ gene, a 600 bp amplicon was obtained on PCR amplification. The *Hph I* RE digestion of PCR product generated fragments of 465, 454 bp. smaller fragments could not be resolved properly. On the basis of presence and absence of restriction fragments birds were grouped into three genotypes i.e. AA, AB, BB. Present investigation revealed only two genotype i.e. AA and AB, in the birds analyzed (Fig.6). AA genotype had only 465 bp fragments whereas AB genotype had both 465 and 454 bp fragments. Genotypic and gene frequencies for IL-2Rγare presented below-

**Table.8. Gene and Genotypic Frequencies of IL-2Rγ**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Gene</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>AB</td>
<td>BB</td>
</tr>
<tr>
<td>Frequency</td>
<td>0.64</td>
<td>0.36</td>
</tr>
<tr>
<td></td>
<td>0.0</td>
<td>0.82</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.18</td>
</tr>
</tbody>
</table>

Thus, PCR-RFLP of 600 bp product of IL-2Rγ gene with *Hph I* restriction enzyme revealed polymorphic banding pattern in Aseel native chicken.
5.2.7. DNA Polymorphism at IL-15Rα gene

For IL-15Rα gene, 246 bp amplicon was obtained on PCR amplification. The Alu I RE digestion of PCR product generated fragments of 123, 80 bp. Smaller fragments could not be resolved properly. On the basis of presence and absence of restriction fragments birds were genotyped and only one genotype i.e. AB, could be observed in all the birds (Fig. 7). AB genotype had both 123 and 80 bp fragments. Genotypic and gene frequencies for IL-2Rγ are presented below-

Table 9. Gene and Genotypic Frequencies of IL-15Rα

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Genotype</td>
</tr>
<tr>
<td></td>
<td>AA</td>
</tr>
<tr>
<td>Frequency</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Thus, PCR-RFLP of 246 bp product of IL-15Rα gene with Alu I restriction enzyme revealed polymorphic banding pattern in Aseel native chicken.

5.2.8. DNA Polymorphism at IFN-γ promoter gene

For IFN-γ promoter gene, a 670 bp amplicon was obtained on PCR amplification. The Tsp 509 I RE digestion of PCR product generated fragments of 168, 123, 104, 99, 88, 64, 54 bp. However fragments of 104, 99 and 104 could not be resolved properly due to small difference
in fragment sizes. Smaller fragments also could not be resolved properly. On the basis of presence and absence of restriction fragments, birds were grouped into three genotypes i.e. AA, AB, BB. Present investigation revealed all the three genotype i.e. AA and AB, in all the birds analyzed (Fig.8). AA genotype had all the fragments viz. 168, 123, 99, 88, 54 bp except 104 and 64 bp fragments. Whereas AB genotype had all the fragments i.e. 168, 123, 104, 99, 88, 64, 54 bp. AB genotype had all the fragments except 168 bp fragment. Genotypic and gene frequencies for IL-2Rγare presented below-

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>A</td>
</tr>
<tr>
<td>AB</td>
<td>B</td>
</tr>
<tr>
<td>BB</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.17</td>
</tr>
<tr>
<td>0.30</td>
</tr>
<tr>
<td>0.53</td>
</tr>
</tbody>
</table>

Thus, PCR-RFLP of 670 bp product of IFN-γ promoter gene with Tsp 509 I restriction enzyme revealed polymorphic banding patterns in Aseel native chicken.

5.3. Association between DNA polymorphism at specific loci and Immunocompetence traits

Genotypic differences were analyzed to association Immunocompetence traits (HA, LZM and IGG) and DNA polymorphism at specific loci.
5.3.1. Association of DNA polymorphism at IL-2Rγ loci with immunocompetence traits

Two genotype were found in case of IL-2Rγ gene. Heterozygotes at IL-2Rγ gene had higher HA titres than the homozygote observed in this study. They revealed higher titre than the population mean too. However Lyozyme level in heterozygotes was lower than that in both homozygotes and overall population.

Table 1.1. Least Square Analysis Population mean of Immunocompetence traits.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Immunocompetence Traits</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HA</td>
</tr>
<tr>
<td>AA</td>
<td>9.71±3.46</td>
</tr>
<tr>
<td>AB</td>
<td>13.3±2.86a</td>
</tr>
<tr>
<td>Population mean</td>
<td>12.38±0.60</td>
</tr>
</tbody>
</table>

Means with dissimilar superscripts in a column under a class differ significantly (P < 0.05)

5.3.2. Association of DNA polymorphism at IL-15Rα gene loci with immunocompetence traits

Only one genotype was found in case of IL-15Rα gene. Heterozygotes at IL-15Rα gene had much lower HA titres than the population average. Average value for HA, Lysozyme and IgG were 10.7±2.5, 3.8±0.41 and 56.75±9.77, respectively. Serum lysozyme level
in heterozygotes was higher than population mean but other two traits were lower than the population mean.

5.3.3. Association of DNA polymorphism at IFN-γ promoter loci with immunocompetence traits

Three genotypes were observed in case of IFN-γ promoter gene. Heterozygotes were superior to both homozygotes and showed higher averages than either of the homozygotes as well as population mean for HA titre and serum Lysozyme levels.

Table 12. Least Square analysis of Mean of genotype of immunocompetence traits.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Immunocompetence traits</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HA</td>
</tr>
<tr>
<td>AA</td>
<td>12.17±2.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>AB</td>
<td>15.22±2.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>BB</td>
<td>7.8±1.5&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Population mean</td>
<td>12.38±0.60</td>
</tr>
</tbody>
</table>

Means with dissimilar superscripts in a column under a class differ significantly (P ≤0.05)
5.3.4. Phenotypic correlations \((r_p)\) among immunocompetence traits

Phenotypic correlations were estimated among the three immunocompetence traits (HA, LZM and IGG) in random bred Aseel native chicken, which were positive and low to medium in magnitude. Phenotypic correlation estimated between HA titre and IgG concentration; HA titre and serum lysozyme activity and serum IgG concentration and serum lysozyme activity were 0.029, 0.063 and 0.099, respectively.

In the current study, associations of DNA polymorphisms in four immune-related genes with antibody response kinetics to SRBC and BA, and BW in an F2 population were determined and the major finding was the association of a novel \(IFNG\) promoter-region SNP with multiple traits of Immune response and with BW in the F2 population.

The current study analyzed a new region within the \(IFNG\) promoter region, to expand knowledge on polymorphisms and effects of SNP in this gene’s promoter.

The \(IFNG\) SNP was associated with 12-wk BW in the M5.1 lineage in the current study, with the birds that were homozygous for the Fayoumi allele being significantly lighter than the other two genotype classes. This genotype also had a more rapid immune response, with
the Fayoumi homozygotes for the IFNG reaching the Tmin to BA significantly faster than the Leghorn homozygotes.

In summary, the current study identified new SNP within the SLC11A1, IFNG, and TLR4 genes and expands previous research on associations of IFNG promoter-region polymorphisms and antibody kinetics. The effect of the IFNG gene polymorphisms on both antibody response kinetics and BW emphasizes the importance of evaluating the effect of genetic variation on multiple phenotypic systems, as negative pleiotropic effects may be deleted.

Identifying the QTL which is responsible for the economic important traits will facilitate poultry breeding programs. Molecular genetic information is required to be used to enhance genetic improvement of animal species. Amplification of IL-2Rγ resolved a 600 bp amplicon in all the samples. Digestion of this amplicon with Hph I RE generated fragments of 465 or 465 and 454 bp sizes.
Fig. 6. PCR-RFLP analysis of IL-2Rγ gene in Aseel native chickens. (A) Amplified product of 600 bp (B) Hph I PCR-RFLP indicates various genotypes. bp1, bp2 indicate molecular sizes of DNA markers and specific
Fig. 7. PCR-RFLP analysis of IL-15Ra gene in Aseel native chickens. (A) Amplified product of 246 bp (B) Alu I PCR-RFLP indicates various genotypes. bp₁, bp₂ indicate molecular sizes of DNA markers and specific amplicon (A) or
Fig. 8. PCR-RFLP analysis of IFN-γ promoter gene in Aseel native chickens. (A) Amplified product of 670 bp (B) Tsp509 I PCR-RFLP indicates various genotypes. bp₁, bp₂ indicate molecular sizes of DNA markers and specific amplicon (A) or RE digests (B), respectively.
6.1. IMMUNOLOGICAL TRAITS

Accordingly three important traits viz. humoral response to sheep RBCs, serum lysozyme activity and serum IgG level were analyzed in Aseel breed of native chicken. Various non-genetic factors affecting these traits were also studied.

6.1.1. Antibody response to Sheep RBC

The antibody titres against sheep RBCs were measured through HA test on 5th dpi. HA titres ranged from 2-21. Average HA titre was 12.80±0.74 and 11.96±0.64 in female and males, respectively. The overall average was 12.38±0.60.

(Saxena et al., 1997) estimated the response to SRBC in Guinea fowl, Kadaknath and broilers on 5th dpi as 1.520±0.487, 1.525±0.068, and 1.386±0.119, respectively.

(Shivakumar, 2003) reported a titre of 8.89±0.29 in the base population of IWG of WLH chicken.

(Sivaraman et al., 2005) observed a mean titer of 6.289±0.246 in SDL broiler chickens.
(Singh, 2005) reported a titer of 12.00±1.48 and 4.46±0.68 in high and low SRBC response lines of IWG-WLH after three generations of divergent selection based on response to SRBC.

In earlier reports also, Aseel has been found to possess better immunocompetence traits than other breeds (Kundu et al., 1999). It has been reported that advanced genetic selection for economic performance parameters reduced the immune status (Yegani, et al., 2005). Survival of the fittest i.e. natural selection has maintained only the immunocompetent population of birds.

Besides, genetic factors (Saxena et al., 1997, Sivaraman et al., 2003) other factors have also been reported to influence the response to sheep RBCs. Higher dose elicited higher response (Ubosi et al., 1985, van der Zijpp 1983a & Boa-Amponsem et al., 2000); intravenous route elicited higher responses than other routes (Boa-Amponsem et al., 2001), age of bird affected the response (Ubosi et al., 1984) etc. (Haunshi et al., 2011). All the four groups of vaccinated birds showed good antibody response. However, no significant differences were observed in PHA titres between groups of birds on different periods of vaccination though it was assumed that there might be significantly higher antibody response in Aseel and its crosses with RIR as Aseel is a very strong, muscular and compact-build bird (Rana et al., 2010).
Another inland study in ducks revealed that Deshi (Indigenous) ducks produce better immune response to fowl cholera vaccine compared to Khaki Campbell and Jiding breeds of ducks.

In this study effect of sex and age on humoral response to sheep erythrocytes measured as HA titre was studied. (Ubosi et al., 1985) has also reported effect of age on HA titre.

The finding is comparable to those reported by (Saxena et al., 1997, Nath 1999, Sivaraman 2004) and (Singh, 2005) in different genetic stock.

Sex had non-significant effect on HA titre in guinea fowl too (Saxena et al., 1997). (Kundu et al., 1999) also could not observe any sex difference for HA titre in Indian and exotic birds. However significant sex differences (females having more titre than males) have been reported in 9th generation of divergent selection based on SRBC response (Pinard et al., 1992). Sexual dimorphism was attributed to the effect of sex hormone on the thymus and the immune cells as seen in mammals (Boa-Amponsem et al., 2001).
6.2. Serum Lysozyme Concentration

The serum lysozyme concentration was estimated using Lysoplate assay method. The least squares ANOVA of serum lysozyme level ranged from 1.23-10.34 μg/ml. Average serum lysozyme level was 3.40±0.23 and 3.44±0.20 in male and females, respectively. The overall average was 3.42±0.19. The mean lysozyme levels observed in the present study were higher than earlier reports in various breeds of chickens. (Saxena, 1993) reported lysozyme concentrations in desi fowl and commercial broilers as 1.79±0.07 and 1.33±0.036 μg/ml, respectively. (Shivakumar, 2003) reported serum lysozyme level in IWG and IWJ lines of WLH chickens as 2.18±0.04 and 1.26±0.04 μg/ml, respectively. (Sivaraman, 2004) observed lysozyme level as 1.86±0.075 μg/ml in broiler chicken. (Nath, 1999) reported comparable lysozyme concentration (3.55 to 5.77 μg/ml) in broiler population. The lysozyme concentrations reported in other poultry species were 1.18±0.03 μg/ml (Saxena, 1993) in guinea fowl, 1.82±0.06 and 1.71±0.06 μg/ml in Black and White turkey varieties, respectively (Singh, 2003).

The influence of sex on serum lysozyme was non-significant (Sivaraman et al., 2005) also reported that sex had non-significant effect on serum lysozyme.
Contrarily, significant effect of sex on serum lysozyme levels were observed in various genetic groups of broiler chicken (Nath, 1999) in synthetic broiler population (Sivaraman, 2003) and significantly higher values of lysozyme were observed in females (1.24±0.03 μg/ml) than males (1.11±0.04 μg/ml) in guinea fowl by Saxena (1993) and Singh et al., (2005) in WLH where it averaged 2.09±0.36 and 2.89±0.36 μg/ml in males and females, respectively. (Shivakumar, 2003) also reported significant sex effect on serum lysozyme level in IWG-WLH chicken; in turkey varieties also, females’ revealed higher titre than males in first generation (Singh, 2003). Present findings suggested that the genetic mechanisms responsible for mounting of antibody responses to sheep RBC and regulation of serum lysozyme levels in Aseel chicken might be independent.

6.3. Serum IgG concentrations

Serum IgG is the most abundant antibody and constitutes approximately 80% of the total immunoglobulins. The bird’s ability to mount antibody responses to other antigens is primarily revealed by serum IgG concentration.
The average serum IgG concentration was 69.87±6.57 (Fig. 4.4). It was higher in males (74.09±8.13) than females (65.33±10.20).

Previous reports on IgG concentrations revealed 7.53±0.61 to 15.99±2.2 mg/ml IgG concentration in White leghorn chicken and 20.51±0.22 mg/ml in indigenous Aseel (Ahrestani et al., 1987).

The serum IgG concentrations in broiler & indigenous birds were reported to be 8.01±0.4 and 10.01±0.4 mg/ml, respectively (Saxena, 1993). Another finding revealed IgG concentration of 13.5±0.68 mg/ml in WLH (Rees & Norskog, 1981).

(Singh, 2005) reported non-significant effect of sex on IgG concentrations. The least squares means for males and females were 59.09±7.69 and 52.08±7.74 mg/ml in WLH, respectively. Present findings of non-significant differences between sexes, but relatively higher titre in males can be supported with the reports of (Van der Zijpp et al., 1983), who also observed significantly higher titre in WPR males than females.
6.4. DNA Polymorphism in IL-2Rγ, IL-15Rα and IFN-γ promoter genes

DNA polymorphism IL-2Rγ, IL-15Rα and IFN-γ promoter were studied using PCR-RFLP. RFLP patterns were analyzed to study the association of genotypes with immunocompetence traits.

6.4.1. PCR optimization

The optimized PCR reaction mixture and PCR programmes gave satisfactory amplification of all the genes in Aseel breed of native chicken.

6.4.1.1. DNA Polymorphism at IL-2Rγ gene

Previous reports revealed the presence of Hph I RE sites in the 600bp of IL-2Rγ which generated the fragments of 465, 64, 40, 31 bp (LL Genotype) and 454, 64, 40, 31, 11 bp (FF Genotype) (Zhou and Lamont 2003).

6.4.1.2. DNA Polymorphism at IL-15Rα gene

The amplified product was double the size of product reported earlier using same set of primers (Zhou and Lamont 2003). Singh also reported similar amplicons in WLH. The Alu I RE digestion of PCR product generated fragments of 123, 80 bp. smaller fragments could not
be resolved properly. Previous reports revealed the presence of \textit{Alu} I RE sites in the 123 bp of IL-15R\textalpha{} which generated the fragments of 123 bp (LL Genotype) and 80, 43 bp (FF Genotype) (Zhou and Lamont 2003).

(Zhou \textit{et al.}, 2001) reported SNP at 425 bp in IL-2 gene between Leghorn and Fayoumi M15.2 Lines. Zhou and Lamont (2003) sequenced a 600 bp product of IL-2R\textgamma{}gene and found T-C SNP between the Leghorn and Fayoumi M5.1 Lines but no polymorphism was found in between Leghorn and Fayoumi M15.2 Lines. Zhou and Lamont, 2003, amplified 123 bp product of IL-15R\textalpha{} gene, which showed a G-A SNP between the Leghorn and the Fayoumi Lines.

(Singh , 2005) observed monomorphic PCR-RFLP patterns in IL-2R\textgamma{}and IL-15R\textalpha{}genes with \textit{Hph} I and \textit{Alu} I restriction enzymes respectively, in divergent WLH lines.

\textbf{6.4.1.3. DNA Polymorphism at IFN-}\gamma{}\textbf{ promoter gene}

Previous reports revealed the presence of \textit{Tsp} 509 I RE sites in the 670 bp of IFN-\gamma{} promoter gene which generated the fragments of 168, 123, 99, 88, 54 bp (LL Genotype) and 123, 104, 99, 88, 64, 54 bp (FF Genotype) (Zhou \textit{et al.}, 2001).
6.5. Association of DNA Polymorphism at IL-2Rγ, IL-15Rα And IFN-γ, Loci With Immunocompetence Traits

(Kumar et al., 2001) also observed low $r_P$ among immunocompetence traits. Similar lower estimates in broiler chicken have also been reported (Sivaraman et al., 2003). (Sivaraman et al., 2005) reported that the phenotypic correlation among immunocompetence traits were not significantly different than zero.

Resistance to most diseases is likely controlled by several genes. Molecular genetics approaches can be used to investigate polygenic control of immune response and disease resistance, and the resulting understanding of the genetic basis of polygenic control of immune response may be utilized to improve chicken health (Lamont, 1998).

In the current study, associations of DNA polymorphisms in four immune-related genes with antibody response kinetics to SRBC and BA, and BW in an F2 population were determined and the major finding was the association of a novel IFNG promoter-region SNP with multiple traits of Immune response and with BW in the F2 population.

There were three SNP detected previously in the IFNG promoter region in the same F2 population as used in the current study, (Zhou et al., 2001). The current study analyzed a new region within the IFNG promoter region, to expand knowledge on polymorphisms and effects of
SNP in this gene’s promoter. Identifying two additional SNP in the 396 bp sequenced showed the IFNG promoter region to be highly polymorphic, much more than the average of 5 SNP per kb between chicken lines (Wong et al., 2004).

Previous studies on the same F2 population demonstrated that a different IFNG promoter SNP than that characterized in the current study was associated with the level of circulating IFNG protein level after both primary and secondary immunization (Zhou et al., 2002). The circulating IFNG protein level was associated with SRBC antibody response traits, suggesting that the expressed protein level may be one mechanism by which IFNG gene polymorphisms modulates the immune response (Zhou et al., 2002). In the same lines as were used to establish the F2 population evaluated in the current study, IFNG mRNA levels were increased in Salmonella enteritidis-infected chicks compared to non-infected chicks, providing additional support for a role of this gene’s expression in immune response (Cheeseman et al., 2006).

A recent study of the effects of an IFNG SNP on various traits in broiler chickens (Ye et al., 2006) demonstrated effects on mortality, and immune-related (infectious bursal disease vaccine antibody) and growth-related (BW at 40 d and feed conversion ratio) traits. Therefore, it was of interest to explore the effect of the IFNG SNP on BW, as well as the antibody traits, in the current study. The IFNG SNP was associated
with 12-wk BW in the M5.1 lineage in the current study, with the birds that were homozygous for the Fayoumi allele being significantly lighter than the other two genotype classes. This genotype also had a more rapid immune response, with the Fayoumi homozygotes for the IFNG reaching the Tmin to BA significantly faster than the Leghorn homozygotes.

In the (Ye et al., 2006) studied the same IFNG SNP allele that was associated with higher BW and lower mortality, was associated with lower antibody response to infectious bursal disease vaccine.

Cumulatively, these studies and others (Siegel and Gross, 1980; Martin et al., 1990; Mashaly et al., 2000) indicate a negative association between the effect of gene polymorphisms on BW and their effects on antibody response parameters, perhaps due to the elevation in metabolic rate of the high immune response genotype.

In summary, the current study identified new SNP within the SLC11A1, IFNG, and TLR4 genes and expands previous research on associations of IFNG promoter-region polymorphisms and antibody kinetics. The effect of the IFNG gene polymorphisms on both antibody response kinetics and BW emphasizes the importance of evaluating the effect of genetic variation on multiple phenotypic systems, as negative pleiotropic effects may be deleted.
Identifying the QTL which is responsible for the economic important traits will facilitate poultry breeding programs. Molecular genetic information is required to be used to enhance genetic improvement of animal species. The candidate gene approach is a very powerful method to investigate associations of gene polymorphisms with economically important traits in farm animals (Rothschild and Soller, 1997). Many studies have examined growth, skeletal and immunity traits using the candidate gene approach in chickens (example, Zhou et al., 2001; Amills et al., 2003; Li et al., 2003).

Amplification of IL-2Ry resolved a 600 bp amplicon in all the samples. Digestion of this amplicon with Hph I RE generated fragments of 465 or 465 and 454 bp sizes. (Zhou and Lamont, 2003) while analyzing IL-2Ry in the crosses of Fayoumi and Leghorn also obtained same size of the amplicon, which upon digestion with Hph I RE yielded fragments of 465, 64,40,31 bp (LL Genotype) and 454, 64, 40, 31, 11 bp (FF Genotype).
Indian breeds of chicken are well known for their resistance to diseases and tropical adaptability. In present investigation, Aseel breed of native chicken was evaluated for immunocompetence traits viz. antibody response to SRBC, serum lysozyme activity and serum IgG level and DNA polymorphism at IL-2Rγ, IL-15Rα and IFN-γ promoter gene along with associations between DNA polymorphism and immunocompetence traits.

Humoral immune response to SRBC was measured through HA test. The serum lysozyme concentration was estimated by lysoplate assay using *Micrococcus lysodeikticus* as substrate. Singal radial immunodiffusion assay was used to estimate the total IgG in serum. The data on immunocompetence traits were analyzed by least square analysis of variance taking age group and sex as fixed effects in the model. HA titre ranged from 2-21. Average HA titre was 12.80±0.74 and 11.96±0.64 in male and females, respectively; overall average being 12.38±0.60. Serum lysozyme level ranged from 1.23-10.34 μg/ml. Age group effect was significant (P≤0.05) on HA titre only. Older birds demonstrated higher titres.
On the basis of HA titre birds were grouped high, medium and low titre group. Polymorphism in these genes were analyzed by PCR-RFLP technique using \textit{Hph I}, \textit{Alu I} and \textit{Tsp509 I} RE, respectively.

Association between DNA polymorphism i.e. PCR-RFLP patterns and immunocompetence traits were analyzed. Heterozygotes of all the three genotypes demonstrated higher magnitude of all the three immunocompetence traits \textit{viz}. HA titre to SRBC, serum lysozyme activity and serum IgG level averaged more than both the homozygotes.

- Aseel demonstrated relatively higher humoral response to Sheep erythrocytes than other chicken breeds.
- Serum IgG level did not vary significantly ($P > 0.05$) between sexes or age groups; although older birds demonstrated higher values.
- It is evident from the above that Aseel breed of native chicken have high immunocompetence status and PCR-RFLP patterns at certain loci related to immune response vary under different genetic backgrounds.
- In conclusion, the avian immune response is a particularly difficult trait for the commercial poultry breeder to select. Due to the relationship between avian immune response and overall mortality, this trait is important.
• Serum lysozyme level was also relatively higher in Aseel. It did not differ between sexes or among age groups. AA genotype of IL-2 gene had high frequency than the heterozygotes. Other homozygote for this region could not be observed in the samples analyzed.

• Only heterozygotes could be observed for IL-15 gene. PCR-RFLP analysis of IFN gene analysis demonstrated all the three genotypes.

• Heterozygotes at IL-2 gene also had higher HA titres than the homozygote observed in this study; it was higher than the population mean.

• Wide variation in the humoral immune responsiveness and important nucleotide polymorphism at certain loci like IL-2Ry can be exploited for development of higher immunotolerant birds through selective breeding.

• Heterozygotes at IL-15 gene had much lower HA titres than the population average. Heterozygote’s (AB) at IFN gene demonstrated higher HA titres than either of the homozygotes.

• Serum IgG level did not vary significantly (P > 0.05) between sexes or age groups; although older birds demonstrated higher values.
- Only heterozygotes could be observed for IL-15 gene.
- AA genotype of IL-2 gene had high frequency than the heterozygotes. Other homozygote for this region could not be observed in the samples analyzed.
- Heterozygotes at IL-2 gene also had higher HA titers than the homozygote observed in this study; it was higher than the population mean.
- Heterozygotes at IL-15 gene had much lower HA titers than the population average.
- PCR-RFLP analysis of IFN gene analysis demonstrated all the three genotypes.
- Heterozygotes (AB) at IFN gene demonstrated higher HA titers than either of the homozygotes.
- The hemolysis–hemagglutination assay described here can be effectively used to characterize and quantify constitutive innate humoral immunity in birds. The assay is highly repeatable and the results are unambiguous. Agglutination and lysis titers vary significantly between species.
• It is evident from the above that Aseel breed of native chicken have high Immunocompetence status and PCR-RFLP patterns at certain loci related to immune response vary under different genetic backgrounds. Wide variation in the humoral immune responsiveness and important nucleotide polymorphism at certain loci like \textit{IL-2Ry} can be exploited for development of higher immunotolerant birds through selective breeding.

• The result of the present findings revealed that individuals were homozygous as well as heterozygous for loci studied and exhibited a moderate to high level of polymorphism.

• The present findings are suggestive of further exploring and utilization of variation observed at the loci studied to carry out association studies targeting the improvement of humoral immune responsiveness in indigenous chicken of the region.

Overall, the present study demonstrated that Aseel breed of native chicken have high immunocompetence status. Varied levels of humoral immune response in Aseel can be exploited for development of higher immunotolerant birds through selective breeding. DNA analysis of IFN gene may serve as genetic marker for higher immune responsiveness.
8- REFERENCES


• Dil, N. and M. A. Qureshi. (2002) Differential expression of inducible nitric oxide synthase is associated with differential Toll-


Immunogenetics, 20: 373-385. IVRI, Izatnagar, India.


• Kogut, M. H., L. Rothwell and P. Kaiser. (2005) IFN-gamma priming of chicken heterophils up regulates the expression of


• Shivakumar, B.M. and Kumar, S. (2005c) Influence of divergent selection for humoral immune response to Sheep RBC on immunoresponsiveness to New Castle Disease vaccine in White


• Tregaskes, CA., Bumstead, N., Davison, TF & Young, JR. (1996) Chicken B-cell marker ChB6 (Bu-1) is a highly glycosylated protein of unique structure. *Immunogenetics*, 44:212-217.


