# IMMUNOCOMPETENCE AND IMMUNITY RELATED GENES EXPRESSION PROFILING IN ASEEL, KADAKNATH AND WHITE LEGHORN CHICKENS

*Thesis*

*Submitted in partial fulfilment of the requirement for the degree of*

**Doctor of Philosophy**

*in*

ANIMAL GENETICS AND BREEDING

*By*
**Dr. Kokate Laxmikant Sambhaji**
Roll No. 1242

**To**
**DEEMED UNIVERSITY**
**INDIAN VETERINARY RESEARCH INSTITUTE**
**Izatnagar – 243 122 (U.P.)**
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DIVISION OF GENETICS AND BREEDING
INDIAN VETERINARY RESEARCH INSTITUTE
(Deemed University)
IZATNAGAR - 243 122, U.P., INDIA

**Dr. Sanjeev Kumar,**
**M.V.Sc., Ph.D.**
*Principal Scientist*
*Division of Avian Genetics & Breeding, CARI*

**Dated:** 01/02/2013

# Certificate

Certified that the research work embodied in this thesis entitled **"Immunocompetence and immunity related genes expression profiling in Aseel, Kadaknath and White Leghorn chickens"** submitted by **Dr. Kokate Laxmikant Sambhaji, Roll No. 1242,** for the award of **Doctor of Philosophy** degree in **Animal Genetics and Breeding** at Indian Veterinary Research Institute, Izatnagar, is the original work carried out by the candidate himself under my supervision and guidance.

It is further certified that **Dr. Kokate Laxmikant Sambhaji, Roll No. 1242,** has worked for more than 30 months in the Institute and has put in more than 300 days attendance under me from the date of registration for the **Doctor of Philosophy** degree in this Deemed University, as required under the relevant ordinance.

Sanjeev Kumar

**(Sanjeev Kumar)**
**Chairman**
**Student's Advisory Committee**

# Certificate

Certified that the thesis entitled, **"Immunocompetence and immunity related genes expression profiling in Aseel, Kadaknath and White Leghorn chickens"** submitted by **Dr. Kokate Laxmikant Sambhaji,** Roll No. 1242, in partial fulfilment of **Doctor of Philosophy** degree in **Animal Genetics and Breeding** at Indian Veterinary Research Institute, Izatnagar, embodies the original work done by the candidate. The candidate has carried out his work sincerely and methodically.

We have gone through the contents of the thesis and are fully satisfied with the work carried out by the candidate, which is being presented by him for the award of **Doctor of Philosophy** of this Institute.

It is further certified that the candidate has completed all the prescribed requirements governing the award of **Doctor of Philosophy** of the Deemed University, Indian Veterinary Research Institute, Izatnagar.

**Signature of the External Examiner**

Name Dr. B.R. Yadav

Date : 16.05.2013

**(Sanjeev Kumar)**
*Chairman*
*Student's Advisory Committee*

Date : 01/02/13

## MEMBERS OF STUDENT'S ADVISORY COMMITTEE

**Dr. Pushpendra Kumar,** Principal Scientist
Division of Animal Genetics, IVRI, Izatnagar .....

**Dr. V.K. Saxena,** Principal Scientist
Division of Avian Genetics and Breeding, CARI, Izatnagar .....

**Dr. (Mrs) Meena Kataria,** Principal Scientist
Division of Animal Biochemistry, IVRI, Izatnagar .....

**Dr. T.K. Goswami,** Principal Scientist
Incharge Immunology Section, IVRI, Izatnagar .....

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Laxmikant

*Date:* 1/2/2013 **(Kokate Laxmikant Sambhaji)**

*Place: IVRI, Izatnagar*

# Abbreviations

| | | |
|---|---|---|
| < | = | less than |
| > | = | greater than |
| ≤ | = | less than equal to |
| ≥ | = | greater than equal to |
| °C | = | degree centigrade |
| ASL | = | Aseel chicken |
| ANOVA | = | Analysis of variance |
| cm | = | Centimeter |
| cM | = | Centi Morgan |
| cm2 | = | Square centimeter |
| conc. | = | concentration |
| dATP | = | Deoxy adenosine triphosphate |
| DCP | = | Di-calcium Phosphate |
| $ddH_2O$ | = | Double distilled water |
| df | = | degree of freedom |
| DNA | = | Deoxy Ribonucleic Acid |
| dNTP | = | Deoxy nucleotide triphosphate |
| dpi | = | days post immunisation |
| Dr. | = | Doctor |
| EDTA | = | Ethylene Di Ammine Tetra acetic acid |
| *eg.* | = | for example |
| *et al.* | = | *et alii* (and others) |
| *etc.* | = | *et cetera* (and so forth) |
| ELISA | = | Enzyme Linked Immune Sorbant Assay |
| Fig. | = | Figure |
| $h^2$ | = | Heritability |
| HA | = | Haemagglutination |
| HI | = | Haemagglutination Inhibition |
| *i.e.* | = | *id est* (that is) |
| IC | = | Immunocompetence |
| IgG | = | Immunoglobulin-G |
| ISI | = | Indian statistical institute |
| IWH | = | Indian White Leghorn H line |
| KAD | = | Kadaknath chicken |
| log | = | Logarithm |
| MD | = | Marek's Disease |
| mg | = | milligrams |
| µg | = | microgram |
| $MgCl_2$ | = | Magnesium Chloride |
| $MHC^2$ | = | Major Histocompatibility Complex |
| min(s) | = | minutes |

| | | |
|---|---|---|
| µl | = | microlitre |
| ml | = | millilitre |
| mm | = | millimeter |
| µM | = | micro molar |
| MSE | = | Mean error sum of squares |
| MSS | = | Mean sum of squares |
| $Na_2Co_3$ | = | Sodium carbonate |
| NaCl | = | Sodium chloride |
| NaOH | = | Sodium hydroxide |
| NCX | = | Negative Control Mean |
| ND | = | New Castle Disease |
| NDV | = | New Castle Disease Vaccine |
| No. | = | Number |
| $^o$ | = | Degree (unit to measure radial angle) |
| PBS | = | Phosphate Buffer Saline |
| PCR | = | Polymerase Chain Reaction |
| pH | = | Concentration of hydrogen ion |
| PCX | = | Positive Control Mean |
| RBC | = | Red blood corpuscles |
| RD | = | Ranikhet Disease |
| $r_G$ | = | Genetic correlation |
| $r_P$ | = | Phenotypic correlation |
| rpm | = | Rotation per minute |
| SDL | = | Synthetic Dam Line |
| SDS | = | Sodium Do-decyl Sulphate |
| SE | = | Standard error |
| sec(s) | = | second(s) |
| S/P | = | samples to positive ratio |
| SRBC | = | Sheep Red Blood Cells |
| SRID | = | Single Radial Immunodiffusion Assay |
| SS | = | Sum of squares |
| Ta | = | Annealing temperature |
| Taq | = | Taq DNA polymerase |
| TBE | = | Tris borate EDTA |
| TBE | = | Tris Boric acid EDTA Buffer |
| $tdH_2O$ | = | Triple distilled water |
| TE | = | Tris EDTA Buffer |
| TEMED | = | NNN'N'tetramethyl ethylene diamine |
| Tris | = | Trihydroxymethyl aminomethane |
| UV | = | Ultra Violet |
| V | = | Volts |
| Vit. | = | Vitamin |
| *viz.* | = | Videlicet (namely) |
| WLH | = | White Leghorn Breed |
| wt. | = | Weight |

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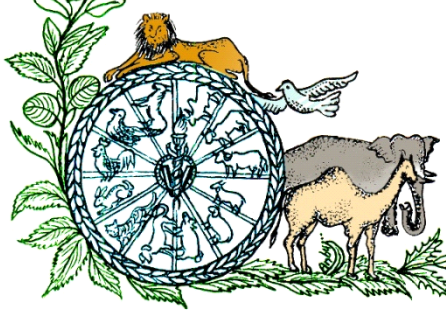
# Introduction



# 1 Introduction

In current intensive poultry production systems, chickens are exposed to a variety of potentially pathogenic organisms. Several approaches have been employed to combat diseases or pathogens including chemotherapy, antibiotics, vaccination biosecurity and managemental investigations. But they have not been proved to be highly efficacious because these approaches suffer with major limitations. Extensive use of drugs leads to the development of Multiple Drug Resistance (MDR) in the infectious agents and drug residues in the egg and meat, which is a cause of concern in the consumers. Because of huge pathogenic exposure, vaccination or antibiotic treatment are not always effective (Alfaro *et al*., 2002). Vaccination is used for immunity induction but it also sometimes leads to morbidity and mortality in vaccinated flock. Use of improved vaccines is associated with the evolution of virulence of many pathogens leading to increased disease losses until new generations of vaccines have been introduced. Vaccination alone cannot manage disease adequately but has to be combined with genetic resistance to exploit the protection against diseases. Genetic selection for improving disease resistance of birds seems to be promising approach (Sivaraman and Kumar, 2006; Gupta *et al.,* 2010). Various selection strategies have been practiced to enhance disease resistance of bird (Sarker *et al.,* 2000; Pinard *et al*., 2004;

Shivakumar *et al*., 2011; Shivakumar and Kumar 2010). Therefore, under the given scenario, probably there is greater need to focus our attention to understand the genetics of diseases and immunity. Breeding for disease resistance is a viable proposition. Progress made per generation is heritable and cumulative over generations. It conserves natural resources and decreases repeated expenditure on health management. Hence, improvement in non-specific genetic disease resistance has been adjudged as one of the best long-term policies.

Immune system plays an important role in protecting poultry against diseases. Better immunocompetence in indigenous chicken is supported by various studies indicating higher disease resistance because of higher complement activity, higher serum lysozyme level and antibody response (Kundu *et al.,* 1999; Chaudhary 2010; Singh *et al.,* 2010; Sivaraman and Kumar, 2010; Jaiswal *et al.,* 2011; Kumar and Kumar, 2011). Introducing indigenous stock in breeding programme could be emphasized in future to improve disease resistance (Zekarias *et al.,* 2002). The non-specific components of immune system such as serum lysozyme concentration play important role in the body's defense against pathogens. Similarly, the specific components of immune system involving humoral response and cell-mediated response are considered to be the important facets of immunocompetence. One of the important non-pathogenic multi-determinant antigens to monitor immune responsiveness in poultry is sheep red blood cell (Siegel and Gross, 1980). Birds eliciting higher antibody response against SRBCs also produce more antibodies to a variety of antigens (Parmentier *et al*., 1998). Lysozyme, an abundant and widespread non-specific bactericidal substance, plays an important role in the body's defense against infection through its direct bacteriolytic action (Biggar and Sturgess, 1977). Immunoglobulin-G is most abundant immunoglobulin in serum

and is regarded as an indicator of general immune response (Pinard *et al.,* 1998). Positive correlations have been found between anti-SRBC response and serum IgG level (Chao and Lee, 2001, Sivaraman *et al.,* 2003). Therefore, understanding of the genetic control of antibody production may provide an opportunity for genetic enhancement of vaccine mediated immunity and thereby resistance to diseases.

Newcastle disease (ND) is one of the most devastating and diseases in poultry. It is an economically important disease of poultry worldwide. The level of immunity influences the severity of ND. Newcastle disease virus (NDV) infects and replicates in avian macrophages, the cells that constitute the first line of immunological defense against infection. It results in immune-suppression, systemic dissemination and enhanced virulence of the virus. Despite the important role in NDV pathogenesis, the molecular changes by NDV infection are not well understood. It is widely recognized that understanding the genetic basis of disease pathogenesis is fundamental to the development of efficient control strategies for NDV and related pathogens. Various studies have been conducted to realize the genetics of resistance to NDV (Tsai *et al.*, 1992; Leitner *et al*., 1994). Still further studies on the resistance mechanism are warranted (Hassan *et al*., 2004).

Host genetics about NDV tolerance is poorly understood. There is a gap in the knowledge about expressed immunity related genes in native breeds, which are believed to be more disease resistant and tolerant as compared to high yielding germplasm. Long term selection for economic traits has led to increase in production traits but loss of resistance to pathogens in high yielding stocks, which might be due to loss of resistant alleles. Hence, a functional genomics assessment of immune related genes is very much needed.

Avian innate immunity provides first line of host defense to microbial infections. Cytokines Interlukin one beta (IL1-β), inducible ni-

tric oxide synthase (iNOS), toll-like receptor (TLR15) is essential effectors molecules of innate and acquired immunity and are crucial signaling molecules in cellular communication. Chicken IL1-β was one of the first chicken cytokines described, which increases antibody production, mediate an inflammatory response. Th1 cytokines (IFN-γ) promotes cell mediated immune response via T cells and macrophages. Interferon γ levels have been correlated with the resistance to several diseases, including MD, IBD, ND (Sadeyen *et al.,* 2006). IFN-γ is involved in macrophage activation and inhibition of viral replication (Djeraba *et al.*, 2002). In many economically important poultry diseases, a primary role of IFN-γ has been suggested (Kaiser, 1996; Plachy *et al*., 1999; Lillehoj and Li., 2004). A protective role of IFN-γ against NDV infection has also been demonstrated (Rautenschlein *et al*., 2000). The level of IFN-γ mRNA expression has been correlated with the level of IFN-γ secretion (Kaiser *et al.*, 2000; Novak *et al.,* 2001; Quere *et al*., 2005). IFN-γ levels are a good measure of cell mediated immunity (Lambrecht *et al*., 2004). The expression of TLR15 mRNA, a novel, avian-specific TLR, was highest in bone marrow, bursa, and spleen and have role in immunity (Higgs *et al.,* 2006). Inducible nitric oxide synthase (iNOS) is an enzyme that produces nitric oxide from the amino acid l-arginine (Alderton *et al.,* 2001). It is produced by macrophages stimulated by cytokines or microbial components. Nitric oxide plays a powerful role in immune responses because of its antimicrobial and anti-tumor functions (MacMicking *et al.,* 1997; Blanchette *et al*., 2003). Expression of the iNOS gene in chickens varies with genetic background (Dil and Qureshi, 2003, Kumar *et al.,* 2011) and also after Eimeria infection (Kim *et al.,* 2008). Understanding the genetics of basal expression levels of genes related to innate immunity is mandatory for effective genetic selection for immune response.

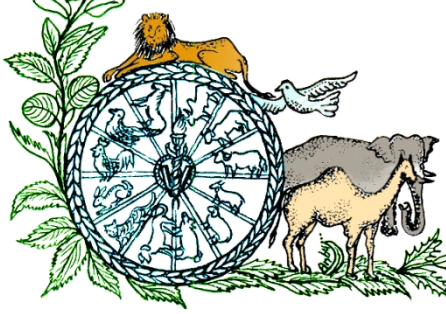
# Introduction...

Aseel and Kadakanath are two important chicken breeds of India. These breeds are being maintained at Central Avian Research Institute and have been characterized for different morphological traits as well as with microsatellite markers. In addition to several unique characteristics, adaptability to tropical environment, higher resistance to diseases as compared to exotic high yielding germplasm make them suitable for studying expression of tropical adaptability and diseases resistance genes, whose identification may pave ways to introgress these characteristics in high yielding germplasm. However, reports on expression patterns in these birds *vis a vis* exotic germplasm are scanty.

Health is an important aspect in poultry rearing as this influences performance and also the cost of production. The input costs on health management have been increasing continuously because of emergence of newer disease. Therefore, identification of tropical adaptability and disease resistance genes would be a novel approach towards introgression of such characters in high yielding germplasm which may lead to decrease in the cost of production. With this background knowledge the present study was undertaken with the following objectives:-

1. Immunocompetence profiling of Aseel, Kadaknath and white Leghorn chicken and to evaluate inheritance pattern of various immunological traits.
2. To determine immune response to ND vaccine in Aseel, Kadaknath and white Leghorn chicken.
3. To study the relative expression of few important immunity related candidate genes in various tissues viz. spleen, thymus and bursa of Aseel, Kadaknath and white Leghorn chicken by quantitative reverse transcription PCR (qRT-PCR).

# Review of Literature



# 2 Review of Literature

## 2.1. Immunocompetence profiling of Aseel, Kadaknath and White Leghorn chicken and to evaluate inheritance pattern of various immunological traits

The immunocompetence status of any breed speaks about its general response to diseases. Resistance to diseases is under the control of certain genes involved in the immune response. 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 titers against SRBC, serum lysozyme level and serum immunoglobulin (IgG) level (Saxena *et al.*, 1997; Shivakumar, 2003; Sivaraman *et al.*, 2003; Singh *et al.*, 2003; Singh, 2005; Kumar, 2006; Chaudhary, 2010; Shivaraman and Kumar, 2010; Singh and Kumar, 2011). Among the most important of all the defensive mechanisms of animals is the immune response. Genetic control of the immune response of an individual consists of three major facets: phagocytosis, T-cell mediated immunity and antibody response. The coordination of these systems enables an individual to resist infection and disease (Lamont, 1991). Divergent selection for antibody production to various antigens in chickens was also effective (Siegel and Gross, 1980).

### 2.1.1. Immunocompetence status in chicken

Immune response to a natural, non-specific, non-pathogenic, T-Cell dependent antigen like Sheep red blood cells (SRBCs) provides an indication of natural immunity status (Van der Zijpp, 1983; Pinard *et al.*, 1992; Saxena *et al.*, 1997). It can be measured through Haemeagglutination (HA) test as described by Van der Zijpp and Leenstra (1980).

#### 2.1.1.1. Antibody response to SRBC

Shukla *et al*. (1996) had also observed significant difference of humoral immune response to SRBC in different pure lines of White Leghorn.

Kundu (1997) studied SRBC response in Aseel, Kadaknath, Naked neck, Frizzle, Dahlem Red, White Leghorn and revealed peak titre on 12 days post immunization (dpi). The HA values were relatively higher for indigenous chicken breeds followed by Dahlem Red and broilers. In most of the breeds, peak response was on 5 dpi and after that a gradual decline was observed.

Singh and Singh (2004) also observed significant difference of SRBC titre between four Indian native breeds namely, Aseel, Kadaknath, Frizzle fowl and naked neck. The least square means ($log_2$ value) of response to SRBC in Aseel (Peela variety) was (11.61) observed. However, Kundu *et al.* (1999) reported lower values of response to SRBC in Kadaknath (5.31) and Aseel (5.10) on $5^{th}$ day post immunization.

Sivaraman *et al.* (2005) studied a synthetic dam line (SDL) of broiler chickens for immunological traits and evaluated their genetic and non-genetic parameters. Least squares means for HA titre was 6.289±0.246. Least squares analysis of variance revealed a significant effect of sire only on HA titre, and no interaction of sire by sex on the trait. Males tended to show a lower response to SRBC than females but

the differences were not significant.

Chatterjee *et al*. (2006) had also observed significant difference of humoral immune response to SRBC in different pure lines of White Leghorn and also obtained moderate heritability of this trait in pure lines of White Leghorn.

In a recent study (Kumar, 2006) in Aseel chicken, the antibody titres against sheep RBCs on 5$^{th}$ dpi averaged 12.80 ±0.74 and 11.96 ± 0.64 in females and males respectively: overall average being 12.38 ± 0.60. Further, older birds had higher mean antibody titres than the younger birds (13.0 ± 2.13 vs 10.88 ± 0.54). The influence of sex on HA titre was statistically non-significant, (Kumar, 2006), although males has higher mean antibody titre (12.80 ± 0.74) than females (11.96 ± 0.64).

Chatterjee *et al.* (2007a) studied two Indian native chickens namely, Kadaknath and Aseel for immunocompetence traits. The humoral immune response to SRBC was significantly (P<0.05) higher in Aseel (77.25 ± 0.18) than Kadaknath (5.70 ± 0.25).

Chatterjee *et al.* (2007b) reported that the humoral immune response to SRBC of inbred and non-inbred populations of Dahlem Red differed significantly (p<0.05) from each other. The immune response to SRBC was highest in non-inbred group (8.79±1.44) followed by full sib mated group (FS) (7.60±1.78) and half sib mated group (HS) (6.23±1.54) and the heritability estimate of SRBC titre was moderate (0.27±0.19).

Deif *et al.* (2007) studied immunocompetence of broiler chicks fed marginal and high dietary protein levels under winter season of Egypt. With respect to immunocompetence, it was noticed that the Hubbard broiler chicks had significantly higher total anti-SRBCs antibody titre compared to Cobb ones. Negative relationship between

body weight and total anti-SRBC antibody titer was observed in all groups.

Saini *et al.* (2007) studied SRBC response in the RIR strains, and found that RIR-C had higher base total HA titre than RIR-B. Among the RIR strains, RIR-C had higher total antibody titre than RIR-B. The highest HA titre at 4 days PPI was recorded in strain RIR-C (5.20) indicating highest production of total antibodies in response to inoculation of SRBC. Between RIR strains, RIR-B and RIRC have same level of total antibodies (4.70).

Singh *et al.* (2009) assessed least squares means of HA titre was 7.49±0.25 in Kadaknath chicks.

Gupta *et al.* (2010) profiled immunocompetence in HSRBC and LSRBC lines of white Leghorn chicken divergently selected for humoral response to sheep erythrocytes. Least squares mean of HA titre 8.06±0.22 in HSRBC and 7.87±0.26 in LSRBC lines. All the traits in both the lines were significantly affected by hatch. A non-significant effect of sex on HA titre in both the lines observed.

Eid *et al.* (2010) evaluated the effect of both strain and sex against of SRBCs antigen on live body weight, at different ages in broiler chickens and found that there was no significant difference in interaction between strain x line, sex x line and strain x sex x line in body weight. However, these interactions were significant ($P \leq 0.05$) at only 7- wk of age.

Rajkumar *et al.* (2010) evaluated the effect of naked neck (Na) gene on immune competence traits in three genotypes (NaNa, Nana and nana) of the naked neck chicken under a tropical climate (Southern India). The humoral response as measured by antibody titre to sheep red blood cells (SRBC) was significantly higher in NaNa (7.00±0.29) than Nana (6.88±0.65) followed by nana (4.62±0.38).

Kumar and Kumar (2011) demonstrated higher immunocompetence status of Aseel native chicken than those reported for most of the other chicken breeds/ poultry species. The least squares mean of HA titre was 12.38±0.60. Influence of sex was not significant on HA titre.

**2.1.1.2. Serum lysozyme level**

Lysozyme plays an important role in the body's defense against infection (Fleming, 1922). Its role as an antibacterial agent is mediated through stimulatory effect on macrophages' phagocytic function (Thacore and Willet, 1966) as well as its direct bacteriolytic action (Biggar and Sturgess, 1977).

Shivakumar (2003) estimated the serum lysozyme concentration estimates in IWG and IWJ genotypes of WLH to be 2.18 ± 0.04 μg/ml and 1.26 ± 0.04 μg/ml, respectively.

Sivaraman *et al.* (2005) studied a synthetic dam line (SDL) of broiler chickens for immunological traits and evaluated their genetic and non-genetic parameters. Least squares means for lysozyme was 1.860 ± 0.047 μg/ml. Least squares analysis of variance revealed non-significant effect of sire, and no interaction of sire by sex on the trait. Males tended to show a lower serum lysozyme than females but the differences were not significant.

Singh *et al.* (2009) assessed least squares means of serum lysozyme was 2.02±0.05 μg/ml in Kadaknath chicks.

Singh *et al.* (2010) estimated least squares mean of serum lysozyme level to be 2.13±0.03 μg/ml in Aseel Chicken. Significant ($P<0.05$) sexual dimorphism was observed in serum lysozyme levels in HSRBC line and influence of sex on serum lysozyme concentration varied in divergent lines which indicated that the genetic mechanisms responsible for mounting of antibody response to sheep RBC and

regulation of serum lysozyme level might be independent (Singh *et al*., 2010).

Gupta *et al.* (2010) profiled immunocompetence in HSRBC and LSRBC lines of white Leghorn chicken divergently selected for humoral response to sheep erythrocytes. Least squares means of serum lysozyme 2.85±0.08 μg/ml in HSRBC and 2.77±0.09 μg/ml in LSRBC lines. All the traits in both the lines were significantly affected by hatch. A non-significant effect of sex on HA titre in both the lines observed.

Chaudhary (2010) reported relatively higher serum lysozyme level in Aseel chicken, wherein the serum lysozyme concentration ranged from 1.90 to 12.82 μg/ml, the average serum lysozyme level being 4.85 ± 0.20 and 4.48 ± 0.22 μg/ml in male and females respectively. The overall average was 4.66 ± 0.16 μg/ml.

Kumar and Kumar (2011) demonstrated higher immunocompetence status of Aseel native chicken than those reported for most of the other chicken breeds/ poultry species. The least squares mean of serum lysozyme was 3.42±0.19 μg/ml. Influence of sex was not significant on serum lysozyme.

**2.1.1.3. Serum IgG Level**

Serum IgG is the most abundant antibody and constitutes approximately 80% of the total immunoglobulin. The bird's ability to mount antibody responses to other antigen is primarily revealed by serum IgG concentration and is traceable in all body fluids. Ahrestani *et al.* (1987) estimated the serum IgG level in different breeds of chicken by Single Radial Immune Diffusion (SRID) method. Aseel (20.51±0.22 mg/ml) demonstrated significantly higher level than WLH (7.53 ±0.22 mg/ml). Further, higher concentrations in first week of age have been ascribed to the presence of maternal antibodies, which reduces as the age advances.

Sivaraman *et al.* (2005) studied a synthetic dam line (SDL) of broiler chickens for immunological traits and evaluated their genetic and non-genetic parameters. Least squares means for serum IgG was 6.287±0.194 mg/ml. Least squares analysis of variance revealed non-significant effect of sire and no interaction of sire by sex on the trait. Males tended to show a higher serum IgG than females but the differences were not significant.

Saini *et al.* (2007) studied SRBC response in the RIR strains, and found that Ig G level was higher in RIR-C (2.03 *vs.* 1.93) among RIR strains and there were non-significant differences between strains. Between RIR strains, RIR-C had higher Ig G titre than RIR-B.

Chaudhary (2010) found average serum IgG concentration as 11.73 ± 0.49 in Aseel and it was higher in females (12.64 ± 0.75) than males (10.82 ± 0.64). The least squares mean of HA titre on 5th day post immunization was 9.22 **±**0.20 in Aseel chicken (Singh *et al*., 2010).

Gupta *et al.* (2010) profiled immunocompetence in HSRBC and LSRBC lines of white Leghorn chicken divergently selected for humoral response to sheep erythrocytes. Least squares means of serum IgG were 33.91±1.68 mg/ml in HSRBC and 31.65±1.28 mg/ml in LSRBC lines. All the traits in both the lines were significantly affected by hatch. A non-significant ($P<0.05$) effect of sex on HA titre in both the lines observed.

The least squares mean of serum IgG level was 10.61±0.25 mg/ml in Aseel chicken (Singh *et al.,* 2010).

Singh *et al.* (2009) assessed least squares means of serum IgG was 10.07±0.20 mg/ml in Kadaknath chicks.

Kumar and Kumar (2011) demonstrated higher immunocompetence status of Aseel native chicken than those reported for most of the other chicken breeds/ poultry species.

They observed higher average serum IgG concentration in males than females. Age and sex of the bird had no significant ($p > 0.05$) effect on serum IgG level, although males revealed higher values.

### 2.1.2. Inheritance pattern of various immunological traits

#### 2.1.2.1. Heritability ($h^2$)

The $h^2$ estimates reported in various chicken populations were of low to medium in magnitude. The $h^2$ for SRBC response was 0.25 in high and 0.232 in low SRBC lines of 10 through 14 generations of divergent WLH chickens (Martin *et al.,* 1990). Pinard *et al.* (1992) estimated the heritability for SRBC response in fowl selected divergently for 8 generations. The estimates of the realized $h^2$ in high line were 0.15 and 0.16 when estimated by regression and animal model, respectively. The corresponding values for low lines were 0.22 and 0.26. The $h^2$ with an animal model in ISA Warren cross selected from 9 generations of divergent selection for antibody response at 5 dpi to primary immunonization were 0.31 ± 0.01 for all lines of 9 generations and for 9$^{th}$ genartion, recorded 0.29 ± 0.04 and 0.36 ± 0.05 in high and low lines, respectively.

Shukla *et al.* (1996) reported that the estimates of $h^2$ for anti-SRBC response antibody titre ($log_2$) at 7 dpi in White Leghorn chicks were 0.35 ± 0.16.

Using paternal half-sib method, Saxena *et al.* (1997) found the $h^2$ estimate of anti–SRBC antibodies as 0.35 ± 0.17 in Guinea fowl. The HSRBC and LSRBC lines of IWG-WLH genotype had $h^2$ estimate of 0.349 ±0.160 and 0.065 ± 0.109, respectively (Shivakumar, 2003). Low estimates of $h^2$ was reported in Taiwan country chicken and noted to 0.056 ± 0.182 (Chao and Lee, 2001) and 0.29 ± 0.20 in broiler chicken (Sivaraman *et al.,* 2003). Sivaraman *et al.* (2005) reported that the immunological traits showed low heritability estimates except for HA

titre where it was moderate in a synthetic dam line of broiler chicken (0.261±0.163). The heritability estimates of various IC traits were very low (0.009±0.059 to 0.049±0.083) in the HSRBC line and could not be estimated in LSRBC line of White Leghorn (Gupta *et al.,* 2010). Heritability estimates were low to medium and associated with high standard errors in Aseel chicken (Singh *et al*., 2010; Singh *et al*., 2011).

The $h^2$ estimate for serum lysozyme level was reported by authors as variable in species but in low to moderate in range. The $h^2$ of egg white lysozyme from sire and dam components of variance were 0.205 and 0.345, respectively (Bessarabov and Kry Kanov, 1985). Kumar *et al.* (2002) reported higher heritability estimates for serum lysozyme in layer chickens. Shivakumar (2003) observed the $h^2$ estimates in IWG and IWJ genotypes of base population were 0.10 ± 0.15 and 0.32±0.23, respectively. In $S_1$ generation of IWG genotype the $h^2$ estimates for LLG in the high and low SRBC lines were 0.003 ± 0.079, 0.274 ± 0.159, respectively. In the high SRBC line of IWG genotype the $h^2$ was not estimable while in low SRBC line it was 0.229 ± 0.118. Similarly low estimates were observed in broiler (0.20 ± 1.18) chicken (Sivaraman *et al.,* 2003) and moderate levels (0.214) with high standard errors in broiler populations (Nath, 1999). Heritability estimates were medium to high but associated with higher standard errors. Heritability estimate of lysozyme were high in Kadaknath chickens (0.622±0.315) (Singh *et al.,* 2009). The $h^2$ estimate for serum lysozyme level was noted to 0.020 ± 0.064 in HSRBC and was not estimable in LSRBC line of White Leghorn (Gupta *et al.,* 2010).

In other poultry species, the estimates were 0.36 ± 0.17 in Guinea Fowl (Saxena, 1993) and 0.063 ± 0.151 to 0.106 ± 0.210 in turkey varieties (Singh, 2003).

The $h^2$ estimates for serum IgG level were moderate (0.307 ± 0.217 to 0.418 ± 0.175) in Taiwan country chicken selected for serum

IgG concentration (Chao and Lee, 2001). The $h^2$ values were 0.048 ± 0.092 and 0.124 ± 0.125 respectively in high and low SRBC lines of S1 generation of IWG genotypes, and 0.014 ± 0.062 in IWJ low SRBC line (Shivakumar, 2003). Similarly, lower $h^2$ estimates of 0.16 ± 0.17 were observed in broiler chicken (Sivaraman *et al.*, 2003). The $h^2$ estimate for serum IgG level was assessed to 0.049 ± 0.083 in HSRBC line of IWG genotype and was not estimable in LSRBC line (Gupta *et al.,* 2010).

### 2.1.2.2. Genetic ($r_G$) and phenotypic ($r_P$) correlations

Wide variability in genetic correlation among IC traits was reported by Kean *et al.* (1994).

The genetic correlation ($r_G$) coefficients between HA titre and serum lysozyme were not estimable in high SRBC line and in low line the value was 0.05 ± 0.78 (Shivakumar, 2003). But in Black & White varieties of turkey, the $r_G$ values were medium to high but were opposite in direction (Singh, 2003). Earlier reports in broilers indicated positive low (0.02 ± 0.58) $r_G$ values (Sivaraman *et al.,* 2003). The standard errors of $r_G$ between immunological traits were also very high and the $r_G$ among serum lysozyme and IgG concentrations did not follow any specific trend, whereas HA titre exhibited positive correlations with the other immunological traits in a synthetic dam line of broiler chicken (Sivaraman *et al*., 2005). The genetic correlation ($r_G$) between HA titre and serum lysozyme was positive but out of range in HSRBC line of IWG genotype, thus not precise (Gupta *et al.*, 2010).

The $r_G$ between HA and serum IgG level in high and low-SRBC lines were positive. In IWJ-high SRBC line, the $r_G$ was not estimable but was -0.16 ± 1.65 in IWJ-low SRBC line (Gupta *et al.*, 2010).

The genetic correlation ($r_G$) between serum lysozyme and serum IgG levels was high and positive in Aseel chicken, although higher standard error associated with these estimates made them less precise (Singh *et al*., 2010 and Singh *et al*., 2011).

The phenotypic correlations (rp) among IC traits were reported generally small and negative (Kean *et al*., 1994). Sivaraman *et al*. (2005) also reported for a synthetic dam line of broiler chicken that the $r_P$ values among immunological traits were not significantly different from zero. Similarly, Sivaraman *et al.* (2005) and Singh *et al*. (2009) reported very low phenotypic correlation among immunocompetence traits. Phenotypic correlations were estimated among the three immunocompetence traits (HA, serum lysozyme and serum IgG levels) in random-bred Aseel native chicken, which were positive and low to medium in magnitude (Kumar, 2006). The corresponding values were very low and not significantly different from zero in Kadaknath chickens (Singh *et al.,* 2009) and Aseel chicken (Singh *et al.*, 2010 and Singh *et al*., 2011). Phenotypic correlations among IC traits in both lines were low (P>0.05) and divergent selection significantly influenced humoral response and serum IgG levels, HSRBC line of WLH demonstrated higher magnitudes (Gupta *et al*., 2010).

The $r_P$ value between HA with serum lysozyme level was positive but very low in magnitude (Shivakumar, 2003). Phenotypic correlation estimated between HA titre and serum lysozyme activity was 0.063 in a random-bred Aseel chicken (Kumar, 2006). The corresponding values were noted to 0.056 in HSRBC line of WLH and the corresponding value in LSRBC line of WLH was estimated to 0.015 (Gupta *et al.*, 2010).

The $r_P$ value between HA with serum IgG level was positive but very low in magnitude (Shivakumar, 2003). Phenotypic correlation estimated between HA titre and IgG concentration was 0.029 in a random-bred Aseel chicken (Kumar, 2006). The corresponding value was predictable to 0.118 in HSRBC line of IWG-WLH genotype and the corresponding value in LSRBC line was anticipated to - 0.16 (Gupta *et al.*, 2010).

The $r_P$ value between serum lysozyme levels with serum IgG level was positive but very high in magnitude (0.42 to 0.72) in all the S1 divergent SRBC lines of IWG genotypes (Shivakumar, 2003). Sivaraman *et al.* (2005) reported that the phenotypic correlation among immunocompetence traits were not significantly different than zero. Phenotypic correlation estimated between serum IgG concentration and serum lysozyme activity were 0.099 in a random-bred Aseel chicken (Kumar, 2006). The corresponding value was recorded to - 0.061 in HSRBC line and the corresponding value in LSRBC line was assessed as - 0.056 (Gupta *et al.*, 2010).

Van der Zijpp *et al.* (1983) suggested that these traits should be combined while selecting for improvement in general immune responsiveness, owing to low and non-significant correlations among IC traits.

## 2.2. To determine immune response to ND vaccine in Aseel, Kadaknath and White leghorn chicken

Newcastle disease (ND) is one of the most important diseases affecting poultry throughout the world (Al-Garib *et al*., 2003). Vaccine immunization is the major measure to prevent ND and has obtained good effect. However, there are still immunity failures in poultry which have become the major problem in immune prevention of this disease. It is generally acknowledged that humoral immunity is the main immunity to Newcastle disease virus (NDV). The immunity to NDV was most commonly evaluated by measuring antibody titer in the sera by Haemagglutination inhibition (HI) test and Enzyme *Linked Immune Sorbant Assay* Test (ELISA test).

### 2.2.1. HI test

The immunity to NDV was most commonly evaluated by measuring antibody titre in the sera by Haemagglutination inhibition (HI) test and

high titer of antibodies were generally accepted as a reliable indicator of flock immunity (Beard and Hanson, 2003).

Allan *et al.* (1978) reported that, if the time interval between primary and secondary vaccination is less than 21 days, the antibodies produced by the first vaccination are likely to interfere with the multiplication of the second dose of vaccine virus.

Nasser *et al.* (2000) recorded significant rise of HI antibody titre following booster dose given three weeks after the first vaccination broiler chicks.

Kafi *et al*. (2003) found the highest HI titres after secondary vaccination with BCRDV chicken.

Pakpinyo *et al*. (2003) observed HI antibody titers of birds vaccinated at 10 days of age were higher than those vaccinated at 1 day old.

Shuaib *et al. (*2003) reported that secondary vaccination yielded HI titres that were significantly higher than the HI titres after single vaccination in broiler chickens.

Yao *et al*. (2004) examined 1554 broiler flocks and 363 hybrid broiler flocks in Taiwan for HI antibody titers for ND. The geometric mean of maternal antibody titers of broilers was 54±3. The antibody titers of broilers at marketing age were 26±4 which were significantly lower than the maternal antibody titers.

Sasipreeyajan (2005) tested Serological response against NDV and found highest response at 2 weeks post vaccination which declined by 3 weeks post vaccination.

Jalil *et al*. (2009) observed significant ($p < 0.01$) decreases in HI titres after 7 days of vaccination in chickens. The Primary vaccination at day 7 followed by a booster dosing at day 28 may be followed for

better immune response and protection against ND in broilers.

Yan *et al.* (2011) were evaluated Antibody titers of sera from post vaccinated experimental chickens by HI test. HI antibody titres to live ND vaccine gradually rose from day 7 post vaccination and peaked at day 21.

### 2.2.2. ELISA test

Cuello *et al*. (2003) studied antibody response of White Leghorn breeders against NDV assessed by ELISA. There was significant increment in the antibody levels after the application of vaccine at 2 months post vaccination. The response to the vaccine stayed in the time until 10 months post vaccination.

Shivakumar (2003) observed that the high SRBC line revealed higher antibody titer to NDV in IWG and IWJ genotypes of WL chicken and the peak titer were absorbed at 14 dpi in IWG line and at 14 & 21 dpi in high and low SRBC lines in IWJ.

Al-Zubeedy (2009) studied antibody titers in One day old broiler chicks from four groups of broiler breeder chickens using ELISA test, he revealed that the vaccination of one day old chicks is very important to enhance the maternal derived antibody response.

Hauslaigner *et al.* (2009) studied through an indirect Newcastle disease virus enzyme-linked immunosorbent assay (ELISA) for waterfowl and evaluated concerning its efficiency and its suitability to monitor the antibody response in Muscovy ducks (*Cairina moschata)* and domestic geese (*Anser anser var. domestica*) following vaccination with a commercial inactivated NDV vaccine for chicken. Statistical comparison of ELISA results with those of the HI test, performed in parallel, provided a positive linear correlation between both tests. However, in the course of seroconversion some differences became evident, especially within the 1x dosage group. Here the highest median of the HI titres is already

reached 3 weeks after vaccination, whereas ELISA results provide a more moderate increase, with the highest median determined on 7 weeks after immunization.

Sivaraman and Kumar (2010) studied NDV vaccine response and observed peak at 14 dpi in both high and low *IC*-index line for primary vaccination to NDV. For secondary response, the peak titre was observed at 42 dpi in high line and 35 dpi in low line.

### 2.2.3. Comparison between HI titres and ELISA titres

Charan *et al.* (1981), Marquardt *et al.*, (1985) research workers published that ELISA can detect high levels of Abs to the vaccine virus and considered accurate and sensitive compared to HI test. The test is therefore, proved more practical, sensitive and rapid for detection of Ab titres against NDV vaccines compared to the HI test. However, HI was confirmed more cheaper than ELISA as no micro plate reader is required in addition to the cost of ELISA kit. Similar finding was also published by Bozorghmehrifardl and Mayahi (2000) who showed that HI test is more economic than ELISA kit used for detection of Ab levels against NDV.

Tabidi *et al.* (2004) were compared the antibody (Ab) titres to the intermediate Newcastle disease virus (NDV) vaccine (Komorov strain) in broiler chicks using haemagglutination inhibition (HI) test and an indirect Enzyme-Linked Immunosorbent Assay (ELISA). Non-consistent pattern in the Ab levels between the two tests was observed. ELISA proved more accurate, sensitive and rapid but less economic than HI test when used for detection of Ab titres against NDV vaccines.

Tariq *et al.* (2010) observed a good correlation between ELISA and HI titers (Correlation Coefficient=0.705205) and there was a statistically significant ($P<0.05$) differences between ELISA and HI test at 95% confidence level.

Cadman *et al*. (2011) in his study concluded that, ELISA and HI testing may be used for the serological detection of exposure of ostriches to NDV. ELISA appears to be a more sensitive test and should therefore be regarded as the currently recommended method to conduct sero surveys for NDV exposure and in the evaluation of efficacies of vaccine protocols.

### 2.3. To study the relative expression of few important immunity related candidate genes in various tissues viz. spleen, thymus and bursa of Aseel, Kadaknath and White Leghorn chicken by quantitative reverse transcription PCR (qRT-PCR)

Several immunity related genes have been reported in the literature. But, a few of them viz., IL-1beta, TLR-15, IFN- $\gamma$, iNOS, have been reviewed and were studied in the proposed study.

Chicken IL-1$\beta$, which belongs to the IL-1 super-family of cytokines, was one of the first chicken cytokines described. Chicken IL-1$\beta$ mediates an inflammatory response and increases antibody production, similar to its mammalian counterpart (Leutz *et al*., 1989; Sterneck *et al.,* 1992). Expression of IL-1$\beta$ gene after parasitic infestation differed between chicken inbred lines disparate for the MHC (Kim *et al.,* 2008). Kumar *et al.* (2011) observed that the expression of IL-1$\beta$ was significantly ($P < 0.05$) affected by sex. Males exhibited higher ($P < 0.05$) IL-1$\beta$ expression than females. Although statistically non-significant, a higher level of expression was also observed in males for all other genes tested.

Toll-like receptors (TLRs) are type I transmembrane proteins that play an essential role in innate immune system (Akira *et al.*, 2001). They are expressed by most immune cells as well as epithelial cells (Iqbal *et al*., 2005; Kogut *et al*., 2005). Toll-like receptors are members of a class of cellular receptors known as pattern-recognition receptors, which recognize evolutionarily conserved molecular motifs (pathogen-associated molecular patterns) of infectious microbes. The expression

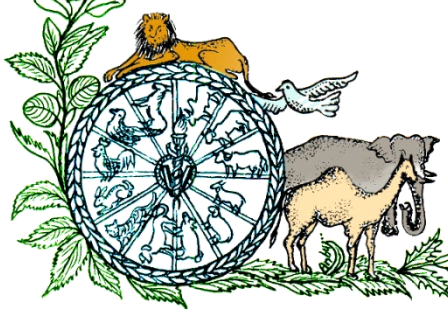
of TLR15 mRNA, a novel, avian-specific TLR, was highest in bone marrow, bursa, spleen, and cecum, and expression was increased in the cecum by infection with S. enterica serovar Typhimurium (Higgs *et al.,* 2006). Expression of TLR15 varies in heterophils from different chicken lines in response to stimulation with Salmonella enterica serovar Enteritidis, but not in nonstimulated cells (Nerren *et al*., 2009).

Kaiser (1996); Plachy *et al*. (1999) and Lillehoj *et al.* (2004) suggested IFN-γ has a primary role in many economically important poultry diseases. Sadeyen *et al.* (2006) observed Interferon γ levels have been correlated with the resistance to several diseases, including MD, IBD, ND. Rautenschlein *et al*. (2000) found a protective role of IFN-γ against NDV infection. Kaiser *et al.* (2000); Novak *et al.* (2001) and Quere *et al*. (2005) reported that the level of IFN-γ mRNA expression has been correlated with the level of IFN-γ secretion. Djeraba *et al*. (2002) found IFN-γ is involved in macrophage activation and inhibition of viral replication. Lambrecht *et al.* (2004) opined that the IFN-γ levels are a good measure of cell mediated immunity. Chicken strains that are resistant to coccidiosis are reported to have high levels of IFN-γ expression (Byrnes *et al.,* 1993; Lowenthal *et al.,* 1997; Yun *et al.,* 2000). Cheeseman *et al*. (2007) examined mRNA expression of Interferon [IFN]-γ, Interleukin [IL]-1β, in the spleen and cecum of day-old chicks of three distinct chicken breeds (broiler, Fayoumi, and Leghorn). They observed breed genetics influencing cytokine mRNA expression.

Inducible nitric oxide synthase (iNOS), also known as NOS-2, is an enzyme that produces nitric oxide (NO) from the amino acid L-arginine (Bogdan, 2001; Bogdan *et al.*, 2000; Alderton *et al.,* 2001). Produced by macrophages stimulated with cytokine and/or microbial components. Bogdan, 2001; Blanchette *et al*., 2003; Bogdan *et al.,* 2000; MacMicking *et al.*, 1997; MacMicking *et al*. (1997) and Blanchette *et al*. (2003) observed that the Nitric oxide plays a powerful role in

immune responses because of its antimicrobial and anti-tumor functions. iNOS activity is primarily regulated at the transcriptional level, although translational and posttranslational events such as protein dimerization and stability along with phosphorylation have been shown to influence iNOS activity (Aktan, 2004; Kleinert *et al*., 2004). Dil and Qureshi (2003) found that expression of the iNOS gene in chickens varies with genetic background. Sundaresan *et al*. (2005) reported that altered expression of iNOS gene in monocyte cell culture after induction with Newcastle Disease virus. Ahmad *et al*. (2007) postulated that the expression of immune related genes like IFN-γ and iNOS are under control of genetic contribution. Kim *et al*. (2008) investigated the influence of genetic differences in the MHC on susceptibility to avian coccidiosis, they found chickens expressed greater levels of transcripts encoding interferon-γ (IFN-γ), interleukin- 1β (IL-1β), IL-15, inducible nitric oxide synthatase and Expression of IL-1β gene after parasitic infestation differed between chicken inbred lines disparate for the MH.

# Materials and Methods



# 3 Materials and Methods

## 3.1. Immunocompetence profiling of Aseel, Kadaknath and White Leghorn chicken and to evaluate inheritance pattern of various immunological traits

### 3.1.1 Experimental animals

**Birds**

A total of 73, 52 and 96 birds belonging to Aseel, Kadaknath and IWH line of WLH chicken respectively at the approximately 6$^{th}$ week of age and being maintained at experimental farms of CARI, Izatnagar, under standard managemental conditions were used in the study.

**Sheep**

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

### 3.1.2 Evaluation of immunocompetence status

The immunocompetence status of experimental birds were evaluated on the basis of three important immunological traits *viz.* humoral immune response to sheep eryrotrocyte assessed by Haemagglutination (HA) test, serum lysozyme concentration estimated by lysoplate method and serum IgG concentration measured by SRID method. The methodology is described below.

### 3.1.2.1 Antibody response to SRBC

The immune response to SRBC was assessed through Haemagglutination (HA) test (Van der Zijpp and Leenstra, 1980) as mentioned below:

***Preparation of sheep RBCs antigen***

Twenty ml of venous 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 4ºC to settle down the RBCs. The RBCs were then washed thrice with PBS (composition given in Appendix I), pH 7.2 by mixing and centrifuging to remove other serum components.

Finally, 1% (v/v) sheep RBCs suspension was prepared by mixing 1 ml of packed sheep RBCs and 99 ml of PBS, which was then used for injection in the experimental birds as an antigen. The suspension was stored at 4ºC.

***Immunization of birds with Sheep RBCs***

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

***Harvesting of immune sera***

Approximately 2 ml of venous blood was collected in sterilized glass tubes on 5$^{th}$ day post immunization (5 dpi) and allowed to clot for 2 - 3 hours at 37ºC. The hyperimmune 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, the clots were broken gently and tubes were centrifuged at 1500 rpm for 1 - 2 min. to collect serum. Sera samples were stored at - 20ºC till further analysis.

***Estimation of antibody titre against sheep RBCs***

The haemagglutination test was carried out as per the following procedure-

- The test was performed in round bottom (U shaped) microtitre plates.
- Firstly 50 µl of the PBS was added in each well.
- Then, 50 µl of serum was added in the first well of each row except the last row where 50 µl of PBS was added; the last row acted as control.
- After thorough mixing, the sera were two-fold serially diluted by taking 50 µl from first well of each row and transferring it in to next well, mixed gently but throughly. This process was continued till last column from where 50 µl solution was discarded.
- Equal volume i.e. 50 µl of 1% SRBC suspension was then added in all the wells followed by thorough mixing by rocking plates on table surface.
- The plates were then incubated at 37ºC for 1 hr in a humid chamber. The number of well with highest dilution (n), which demonstrated complete agglutination (button shaped clumping of RBCs indicating haemagglutination reaction), was recorded as titre and expressed as $\log_2 n$.

**3.1.2.2. Estimation of serum lysozyme concentration**

The serum lysozyme concentration was estimated using Lysoplate assay (Lie *et al*., 1986) method as described below:

***Reagents and chemicals required***

- Dibasic buffer 0.066 M, pH 6.3 (composition given in Appendix I)
- *Micrococcus lysodiekticus*
- Standard Lysozyme

- Agarose
- Agar

***Procedure***

- The lysozyme standards were prepared by dissolving 2 mg of standard lysozyme (SRL, India) in 1 ml of dibasic buffer.
- Serial dilutions were made to get the final concentration of lysozyme as 40 g / ml, 20g / ml, 10g / ml, 5.0 g / ml and 2.5 g / ml.
- The agar lysoplate was set up on a perfect horizontal surface. The glass plate was cleaned and sterilized with spirit and air-dried. The size of the gel was determined on the basis of number of samples to be analyzed. The borders were prepared by 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 following formula:

  Volume (ml) = Length (cm) X Width (cm) X 0.5 cm

- About 50 ml of 1% Agarose in dibasic buffer was sufficient for 10 X 10 $cm^2$ plate.
- After boiling the Agarose in dibasic buffer it was cooled to 60ºC and the prediluted *Micrococcus lysodieketicus* (Sigma, USA) (50 g 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, allowed to spread uniformly and was left at room temperature for polymerization.
- After polymerization of gel, the wells were punched at a distance of approximately 1.5 cm with the help of a gel punch.
- Ten µl of serum sample was loaded in each well.

- Lysozyme standards were also loaded in the wells at one side (4 - 5 dilutions).
- The plate was incubated at 37ºC in humidity-controlled chamber for 24 h.

***Staining and Destaining of Lysoplate***

Plate was stained with 0.2% Coomassie Brilliant Blue (CBB) for 6 hours and excess stain was removed using destaining solution (methanol 150ml, acetic acid 35 ml, distill water ad to 1000 ml). The diameters of the lysed zones were measured with Digital Vernier calipers. The plates were photograph for documentation.

***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 estimated using following regression equation:

$$Y = bx + c$$

Where,

Y = Concentration of lysozyme in unknown sample
b = Slope of regression equation
c = Intercept of regression equation
x = Diameter of the lysed zone around sample.

### 3.1.2.3. Estimation of serum IgG concentration by Single Radial Immunodiffusion (SRID) Assay

Chicken serum IgG neutralizes the antichicken IgG. A 3% (w/v) agarose gel was used as solidifying base to assay IgG concentrations through Single Radial Immunodiffusion (SRID) assay (Mancini *et al.,* 1965) as per following procedure:

***Procedure***

- Clean and sterilized glass plate was placed on smooth leveled horizontal surface.
- The borders prepared with glass strips were sealed with 1 % agar.
- Approximatly, 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 the second half 1.750 ml of anti-chicken IgG (Sigma, USA) was added and after thorough mixing, it was kept at 50ºC in a water bath.
- The temperature of first half was brought down to about 50ºC and second half was mixed. The whole content was then poured on to the glass plate.
- The gel was allowed to solidify for 1 - 2 hour.
- Then, wells were punched at a distance of 1.5 cm with the help of gel punch.
- The standards of chicken IgG (Sigma, USA) *viz.* 25mg/ml, 12.5mg/ml, 6.25mg/ml, 3.125mg/ml and 1.562mg/ml, prepared by serial dilution of stock solution were loaded in the wells to plot standard curve.
- Then 5 µl of unknown sera was diluted to 4 times with 0.1 M Tris and from this 10 µl of each sample was loaded in the wells.
- The plate was incubated at 37ºC for 24 h in humid chamber.

***Staining and Destaining***

- Plates were stained with 0.2% Coomassie Brilliant Blue (CBB) for 6 hr and excess stain was removed with destaining solution.

- The diameters of the precipitation ring around standard as well as unknown samples were measured with the help of Digital Vernier Calipers.
- The plates were photograph for documentation.

***Determination of IgG concentration***

The serum IgG concentrations in unknown samples were determined with the help of regression equation obtained by plotting $\log_2$ concentrations of IgG standards against diameter of the precipitation ring. The slope of the curve and intercept was determined. The IgG 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 precipitation ring around unknown sample.

**Statistical analysis:**

The data generated on immunocompetence traits was analyzed by Least-squares analysis of variance using following models: -

Model 1: $Y_{ijk} = \mu + S_i + H_j + e_{ijk}$ (within breed)

Model 2: $Y_{ijkl} = \mu + G_i + H_j + S_{li} + e_{ijkl}$ (between breeds)

Where,

Y = value of a trait measured on $ijk^{th}$ individual

$\mu$ = Overall mean

$S_i$ = Random effect of $i^{th}$ sire

$H_j$ = fixed effect of $j^{th}$ sex

$G_i$= fixed effect of $i^{th}$ genotype

$S_{li}$ = Effect of $l^{th}$ sire within $i^{th}$ Genotype

$e_{ijk}$ and $e_{ijkl}$ = random error associated with mean '0' and variance $\sigma^2$

Genetic parameters were estimated using paternal half sib (PHS) method.

### 3.2. To determine immune response to ND vaccine in Aseel, Kadaknath and White leghorn chicken

**3.2.1.Experimental birds**: A total of 64, 72 and 80 birds belonging to Aseel, Kadaknath and IWH line of WLH chicken whose sera were collected at different time points were utilized to study immunoresponse to ND vaccine using HI test and sera samples from 40 birds from each of the three genetic group were utilized in ELISA test.

**3.2.2.Vaccination of birds**: the day-old chicks were vaccinated with a dose of $10^{6.5}$ $EID_{50}$ of ND RDF-1 strain through occulo-nasal route and booster was given at 28 days of age.

**3.2.3.Collection of serum samples**: sera were collected on 7, 14, 21, 28, 35 and 42 days post-immunization and stored at -20º C until used.

**3.2.4. Estimation of antibody titer to ND vaccine**: Antibody titers were estimated by HI and ELISA at different time points post vaccination.

#### 3.2.4.1. *Haemagglutination Test* (HI test)

**Materials**

- Normal saline: 0.85 g sodium chloride in 100 ml of distilled water.
- Chicken erythrocytes suspension (1%): venous blood was collected from 6-week-old chickens in anticoagulant. The erythrocytes were

washed thrice by gentle centrifugation in sterile normal saline at 1500 rpm for 5 min. One percent suspension was prepared by adding 99 ml of sterile normal saline to 1 ml of the packed erythrocytes and stored at $4^0$C.

- Microtitre plates – 'V' bottom.
- Micropipette and tips– 25µl
- Antigen: Allantonic fluid collected from Embryonated eggs infected with Newcastle disease virus.

**Procedure**

- Firstly, 25µl of normal saline was added in all the wells in the first row.
- Then 25µl of the antigen (virus containing allantoic fluid) was added in the first well and serial two –fold dilution was made. Twenty five µl of the suspension from the eleventh well was discarded.
- Then 25µl of 1% chicken RBCs were added in all the 12 wells. The last well was taken as RBC control.
- Plate was gently shaken and kept for 20 min.
- Lattice formation i.e. Haemagglutination was observed. The end point is the reciprocal of the highest dilution showing 100% Haemagglutination. The control well showed button formation.
- Calculation of 4HA

4HA=HA titre/4

e.g. if HA titre is 1 in 128.

Then, 4HA=128/4 =32

Therefore, 1ml of the virus infected allantoic fluid +31 ml of the sterile normal saline was a suspension that has 4HA in every 25µl. This was used in HI test.

***Haemagglutination - Inhibition (HI) test***

**Procedure**

- Firstly, 25µl of normal saline was added in all the wells in the first row.
- 25µl of serum was added in the first well and serial two – fold serial dilutions were made. From last well 25µl of the suspension was discarded.
- In this, 25µl of virus suspension containing 4 HA units was added to all the wells and plate was kept for 20 min at room temperature.
- Then, 25µl of 1% chicken RBC suspension was added to all the wells and kept for 45 min at 37º C.
- RBC control: 25µl of saline was added to a well to which only 25µl of 1% chicken RBC was added.
- Titration: - 25µl of saline was added to 4 wells in a row. 25µl of the 4HA virus suspension was added to the first well and two – fold serial dilutions were made. Twenty five µl of the suspension from the fourth well was discarded. Then 25µl of 1% chicken RBC was added to all the four wells and plate was kept for 20 min. The first two wells showed Haemagglutination if the viral suspension prepared is having 4HA units of virus in every 25µl.
- The end point or HI titre of the serum is the reciprocal of the highest dilution showing 100% inhibition of Haemagglutination.

**Statistical analysis:**

Statistical analysis of immune response data to ND vaccine using HI test were expressed as mean ± SE. Data were compared by analysis of variance using the statistical software program SPSS16.0. Significant differences among means were determined by Tukey's HSD test at $P < 0.05$.

### 3.2.4.2. Enzyme Linked Immune Sorbant Assay (ELISA) Test

**Preperation of samples**

Diluted test sera samples to five hundred fold (1:500) with sample diluent prior to assay by diluting 1µl sera of sample with 500 µl of sample diluent). Control were not diluted. Following test procedure was followed.

**Test procedure**

The reagents were at room temperature so that they come to room temperature ($18^0$-$25^0$ C) and then mix gently by inverting and swirling.

- The antigen coated plate and the sample position was recorded.
- Dispensed 100 µl of undiluted nagative control into well $A_1$ and $A_2$.
- Dispensed 100 µl of undiluted positive control into well $A_3$ and $A_4$.
- Dispensed 100 µl of diluted samples into aprropriate wells.
- Incubated for 30 minute at room temperature ($18^0$-$25^0$ C).
- Aspirated liquid content of all wells into appropriate waste reservior.
- Washed each well with approximatly 350 µl of distiled or deionized water 3-5 times. Aspirate completely.

- Dispensed 100 µl of (Goat) Antichicken: Horseradish Peroxidase Conjugate into each well.
- Incubated for 30 minute at room temperature ($18^0$-$25^0$C).
- Repeated steps 6 and 7
- Dispensed 100 µl of TMB substrate solution into each well.
- Incubated for 15 minute at room temperature (18-$25^0$C).
- Dispensed 100 µl of stop solution into each well.
- Measured dand recorded absorbance values at 650nm, i.e. $A^o$ (650).

**Recording of Results**

For the assay to be valied, the difference between the positive control mean and negative control mean (PCX-NCX) was greater than 0.075. The negative control mean absorbance was less than or equal to 0.150. The presence or absorbance of antibody to NDV is determined by relative A (650) value of unknown to the positive control mean. The positive control was standerized and represented significant antibody levels to NDV chicken serum. The relative level of antibody in the samples was determined by calculating the samples to positive (S/P) ratio. Endpoint titres were calculated using the equation described in the calculations section.

**Interpretation of results**

Serum samples with S/P ratios of less than or equal to 0.20 were considered negative. S/P ratio greater than 0.20 were considered positive and indicated vaccination or other exposure to NDV.

**CALCULATIONS**

Negative Control Mean (NCX) = [well A1 (650) + well A2 (650)]/2

Positive Control Mean (PCX**) =** [well A3 (650) + well A4 (650)]/2

S/P Ratio = (sample mean – NCX)/ PCX – NCX

**Titre related S/P at a 1:500 diluation to an end point titre as below:**

$Log_{10}$ Titre = 1.09 ($Log_{10}$ S/P) + 3.36

**Statistical analysis:**

Statistical analysis of immune response data to ND vaccine using ELISA test were expressed as mean±S.E. Data were compared by analysis of variance using the statistical software program SPSS16.0. Significant differences among means were determined by Tukey's HSD test (Jason, 1996) at $P < 0.05$.

## 3.3. To study the relative expression of few important immunity related candidate genes in various tissues viz. spleen, thymus and bursa of Aseel, Kadaknath and White Leghorn chicken by quantitative reverse transcription PCR (qRT-PCR)

### 3.3.1. Experimental Birds

Four birds from each of the three breeds (2 Male and 2 Female to study sex influence) were studied in this experiment. Their HI titres and ELISA titres presented in the Table 3.3.1.1 and Table 3.3.1.2.

**Table 3.3.1.1 HI Antibody titres ($log_2$) of birds used in gene expression in Aseel, Kadaknath and White Leghorn chicken**

| Breeds | Sample No. | Sex | 7 dpi | 14 dpi | 21 dpi | 28 dpi | 35 dpi | 42 dpi |
|---|---|---|---|---|---|---|---|---|
| Aseel | 1 | Male | 6.32 | 6.32 | 6.32 | 7.32 | 7.32 | 8.32 |
| Aseel | 2 | Male | 5.32 | 8.32 | 7.32 | 8.32 | 9.32 | 8.32 |
| Aseel | 3 | Female | 9.32 | 7.32 | 7.32 | 8.32 | 8.32 | 8.32 |
| Aseel | 4 | Female | 6.32 | 6.32 | 7.32 | 7.32 | 7.32 | 9.32 |
| Population mean (Aseel) | 64 (n) | Combined sex | **8.13** | **7.90** | **7.94** | **8.29** | **8.40** | **8.93** |
| Kadaknath | 5 | Male | 7.32 | 7.32 | 8.32 | 8.32 | 8.32 | 9.32 |
| Kadaknath | 6 | Male | 7.32 | 7.32 | 9.32 | 9.32 | 8.32 | 9.32 |
| Kadaknath | 7 | Female | 7.32 | 8.32 | 7.32 | 9.32 | 9.32 | 9.32 |
| Kadaknath | 8 | Female | 6.32 | 7.32 | 7.32 | 8.32 | 8.32 | 9.32 |
| Population mean (Kadaknath) | 72 (n) | Combined sex | **7.32** | **7.74** | **7.56** | **7.86** | **8.43** | **9.14** |
| WLH | 9 | Male | 9.32 | 9.32 | 10.32 | 10.32 | 10.32 | 9.32 |
| WLH | 10 | Male | 10.32 | 7.32 | 10.32 | 9.32 | 8.32 | 10.32 |
| WLH | 11 | Female | 10.32 | 11.32 | 11.32 | 10.32 | 10.32 | 10.32 |
| WLH | 12 | Female | 10.32 | 10.32 | 10.32 | 10.32 | 10.32 | 10.32 |
| Population mean (WLH) | 80(n) | Combined sex | **8.48** | **8.02** | **8.29** | **8.14** | **7.68** | **7.73** |

**Table 3.3.1.2 ELISA Antibody titres ($log_{10}$) of birds used in gene expression in Aseel, Kadaknath and White Leghorn chicken**

| Breeds | Sample No. | Sex | 7 dpi | 14 dpi | 21 dpi | 28 dpi | 35 dpi | 42 dpi |
|---|---|---|---|---|---|---|---|---|
| Aseel | 1 | Male | 2.98 | 2.52 | - | 3.21 | 1.30 | 2.06 |
| Aseel | 2 | Male | 2.18 | 0.00 | 2.31 | 2.27 | 2.22 | 3.22 |
| Aseel | 3 | Female | 1.98 | 1.77 | 1.98 | 2.58 | 2.63 | 3.34 |
| Aseel | 4 | Female | 2.24 | 1.89 | 2.41 | 3.15 | 3.09 | 3.45 |
| Population mean (Aseel) | 40 (n) | Combined sex | **2.13** | **2.05** | **2.43** | **2.80** | **2.82** | **3.04** |
| Kadaknath | 5 | Male | - | - | - | 1.39 | 3.16 | 2.94 |
| Kadaknath | 6 | Male | 2.57 | 2.33 | 2.75 | 2.51 | 2.57 | 2.60 |
| Kadaknath | 7 | Female | 1.81 | 2.43 | 1.95 | 2.28 | 2.60 | 2.71 |
| Kadaknath | 8 | Female | - | 2.31 | 2.16 | 2.72 | 2.01 | 3.40 |
| Population mean (Kadaknath) | 40 (n) | Combined sex | **1.75** | **1.54** | **1.77** | **2.15** | **2.60** | **2.66** |
| WLH | 9 | Male | 3.15 | 2.83 | 2.29 | 2.92 | 3.05 | 3.00 |
| WLH | 10 | Male | 3.40 | 3.52 | - | 1.97 | 2.78 | 2.45 |
| WLH | 11 | Female | 3.39 | 3.28 | 2.16 | 2.49 | 3.01 | 3.06 |
| WLH | 12 | Female | 3.16 | 2.87 | 4.44 | 2.59 | 2.89 | 4.55 |
| Population mean (WLH) | 40 (n) | Combined sex | **2.95** | **2.92** | **2.10** | **2.03** | **2.41** | **2.51** |

**3.3.2. Tissue collection:** Bursa, spleen and thymus from above identified birds were collected at the age of about 6 weeks and stored at -80ºC until further use.

### 3.3.3. Template Synthesis

#### 3.3.3.1. Sterilization and Inactivation of RNases

Glasswares used for RNA work were sterilized in hot air oven at 180$^0$C for at least 5 hours to make them RNase free. All the plastic wares used for the same purpose were thoroughly treated with 0.1% Diethyl pyrocarbonate (DEPC) at 37$^0$C for about 12 hours in order to destroy the RNases and then were sterilized by autoclaving.

#### 3.3.3.2. Isolation of total RNA

- Chopped tissue (Bursa, Spleen and Thymus) sample (15 gm) was taken in 2 ml RNase free micro-centrifuge tubes containing 800 µl TRIzol denaturing solution.
- The tissue was homogenized using tissue homogenizer (polytron, Kinematica AG, Switzerland) at 20000 rpm for about 30 seconds till a uniform suspension is formed.
- One hundred and twenty microliter (160 µl) of chloroform (molecular biology grade) was added into the tube and was thoroughly mixed.
- The tissue suspension was centrifuged at 13500 rpm for 15 min at 4$^0$C in a micro-centrifuge (KUBOTA-3500, Japan).
- The top aqueous phase (containing RNA) was removed and transferred carefully into a fresh 1.5 ml RNase free micro-centrifuge tube without disturbing pellet.
- Equal volume of isopropanol was added to the aqueous phase and incubated at room temperature (25$^0$C) for 20 min to precipitate the RNA.

- The RNA was pelletted by spinning at 10000 rpm for 10min at 4$^0$C; thereafter the isopropanol supernatent was decanted carefully without disturbing the pellet.
- The pellet was washed with 0.5 ml of 70% ice-cold ethanol and the tube was centrifuged at 10000 rpm for 10 min at 4$^0$C.
- The ethanol was decanted and the RNA pellet was air dried in an RNase free environment.
- The air dried RNA pellet was re-suspended in 50 µl of nuclease-free water.

### 3.3.3.3. RNA Quantification

The purity and concentration of the total RNA was checked using nanodrop (NanoDrop 1000, Thermo Scientific, Singapore). The purity of the total RNA was confirmed by considering the ratios of OD values at 260 and 280nm. The purity was further checked by 2 % Agarose gel electrophoresis. The RNA showing any contamination with DNA was incubated with RNase-free DNase (Biogene, CA, USA) at 37°C (@1 unit for 1µl). The DNase was subsequently inactivated by incubation at 65°C for 10 min. Each DNase-treated total RNA sample was estimated and all the samples were adjusted to equal concentration (5.0µg/µl).

### 3.3.3.4. First-strand cDNA synthesis

The first-strand cDNA synthesis was performed using 200 µl PCR microtubes in a thermal block using *Revert Aid*™ first strand cDNA synthesis kit (MBI, Fermentas).

- Eleven micro-liters (11 µl) diluted total RNA (containing 5.0 µg of RNA) was taken in RNase free PCR tube (0.2 ml).
- One micro-liter (1 µl) of random hexamer primer (0.5 µg/µl) was added to each RNA samples and spinned down for few seconds.
- Each tube containing 12 µl of reaction mixture was incubated at 65$^0$C for 5 minutes in thermal cycler.

- Eight micro liters of master mix containing 5X reaction buffer (4 µl), 10mM dNTP mix (2 µl), Ribonuclease inhibitor (1 µl) and Revert Aid™ reverse transcriptase (1 µl) was added to each tube and mixed thoroughly.
- The total reaction mixture was incubated in the thermal cycler according to the manufacturer's instructions i.e. 25$^0$C for 5 min and 42$^0$C for 60 minutes.
- Finally, the reaction was stopped by heating at 70$^0$C for 10 min and the resultant first-strand cDNA was stored at -20$^0$C till use.

### 3.3.4. Genes taken for the study

Four (IL1-β, IFN- γ, iNOS and TLR15) immunity related genes were assessed and β-actin was gene used as Reference gene. Repeated atempts to study IL-2 gene did not yield satisficatory products, details of genes given in the Table 3.3.4.

**Table3.3.4. Oligonucleotide primer sequences used in the real-time PCR for the gene expression study**

| Target gene | Primer set 5'-3' | Prod. size | Reference |
|---|---|---|---|
| **IL1-β** | F CGCTCACAGTCCTTCGACATC<br>R CCGCTCATCACACACGACATGT | 230 | Higgs *et al.* (2006) |
| **IFN-γ** | F- ACAAGTCAAAGCCGCACATC<br>R- TGGATTCTCAAGTCGTTCATCG | 133 | Higgs *et al.* (2006) |
| **iNOS** | F TGGGTGGAAGCCGAAATA<br>R GTACCAGCCGTTGAAAGGAC | 241 | Hong *et al.* (2006) |
| **TLR15** | F GTTCTCTCTCCCAGTTTTGTAAATAGC<br>R GTGGTTCATTGGTTGTTTTTAGGAC | 262 | Higgs *et al.* (2006) |
| **β-actin** | F GGA AGT TAC TCG CCT CTG<br>R AAA GAC ACT TGT TGG GTT AC | 114 | Higgs *et al.* (2006) |

### 3.3.3.6. GENE EXPRESSION STUDY

The relative expression of specific gene mRNA was quantified by real-time PCR detection system (Strata-gene Mx3000p system). All reactions were performed in nuclease-free 8-tube strips with optically clear flat caps (Axygen Scientific, Inc. USA).

**Real-time Polymerase Chain Reaction**

**Reaction Mix (20 µl) & RT-PCR amplification**

Following reaction mix of 20 µl was prepared using SYBR Green PCR master mix (2X DyNAmo™ HS, Finnzymes, USA) which consisted of Meteor *Taq* hot-start DNA polymerase, dNTPs including dUTP and $MgCl_2$ with 4 mM final concentration in optimized buffer components:

| | | |
|---|---|---|
| 1. | 2 X SYBR Green PCR master mix | 10 µl |
| 2. | 10pM Forward Primer | 0.5 µl |
| 3. | 10pM Reverse Primer | 0.5 µl |
| 4. | Nuclease-free Water | 7.5 µl |
| 5. | Diluted (1:10) cDNA | 1.5 µl |

Real-time PCR cycling conditions were, initial denaturation at 95$^0$C for 5 min followed by 40 cycles of denaturation at 95$^0$C for 20s, annealing at 56$^0$C (for IL1-β, IFN-γ and TLR15 genes) or 58$^0$C (for iNOS gene) for 20s and extension at 72$^0$C for 20s. For each sample a dissociation curve (melt curve) was generated after completion of amplification. A negative control containing all the ingredients except cDNA template (non-template control; NTC) was set up invariably for each master mix made for conducting the reactions. β-actin was used as reference genes.

**Preparation of standard curves**

In order to prepare standard curve, a pooled cDNA sample was prepared by taking equal volume, say 2.5 µl, from of the test samples,

and mixed thoroughly but gently. Five standard template samples were prepared from pooled template (cDNA) by 10-fold serially diluting it. These standards were put along with test cDNA template for housekeeping gene (β-actin) as well as for test gene, viz., IL1-β, IFN-γ, iNOS and TLR15. Ct values were plotted against template concentration, starting from lowest to highest. Regression analysis of the standard curve was used to calculate the slopes of the gene-specific $\log_{10}$ dilutions series. R-square values for fit of the regression equation were obtained.

The mean Ct values of test genes were normalized with housekeeping (β-actin) gene using the following formula and the data were transformed and expressed as the adjusted Ct (cycle threshold) values:

Adjusted Ct= 40-[(mean of test gene Ct) + (median of β-actin Ct-mean of β-actin) X (test gene slope / β-actin slope)] (Cheesman *et al.* 2008).

**Statistical analysis**

The data analysis of gene mRNA expression was performed with an ANOVA model using JMP software (ver 9.1) of SAS Institute, 2008.

Following models were used:

**Model A**: Analysis of variance of mRNA expression of four genes in each of the three tissues using genotype and sex as fixed effects **for each tissue**

$$Y_{ijk} = \mu + GG_i + S_j + e_{ijk}$$

Where,

Y = value of mRNA expression on $ijk^{th}$ individual

μ = Overall mean

$GG_i$ = fixed effect of $i^{th}$ genotype

$S_j$ = fixed effect of $j^{th}$ sex

$e_{ijk}$ = random error associated with mean '0' and variance $\sigma^2$

**Model B**: Analysis of variance of mRNA expression in each of the three genotypes using sex and tissue as fixed effects **for each gene**

$$Y_{ijk} = \mu + S_i + T_j + e_{ijk}$$

Where,

Y = value of mRNA expression on $ijk^{th}$ individual

$\mu$ = Overall mean

$S_i$ = fixed effect of $i^{th}$ sex

$T_j$ = fixed effect of $j^{th}$ tissue

$e_{ijk}$ = random error associated with mean '0' and variance $\sigma^2$

**Model C**: Analysis of variance of mRNA expression in each of the three tissues using gene and sex as fixed effects **for each genotype**

$$Y_{ijk} = \mu + S_i + T_j + e_{ijk}$$

Where,

Y = value of mRNA expression on $ijk^{th}$ individual

$\mu$ = Overall mean

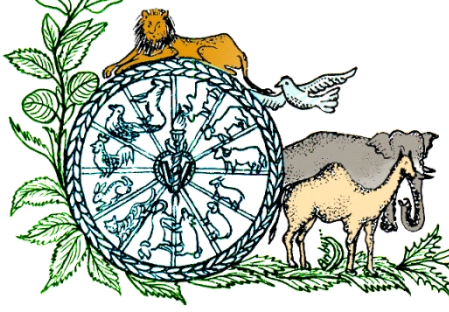
$S_i$ = fixed effect of $i^{th}$ sex

$T_j$ = fixed effect of $j^{th}$ tissue

$e_{ijk}$ = random error associated with mean '0' and variance $\sigma^2$

The results of analysis of variance were presented in the form P-values and wherever any effect was found significant. The average adjusted Ct values were compared using Tukey's HSD test and superscripts were put accordingly.

# Results and Discussion



# 4 Results and Discussion

## 4.1. Immunocompetence profiling of Aseel, Kadaknath and White Leghorn chicken and to evaluate inheritance pattern of various immunological traits

The immunocompetence status of Aseel, Kadaknath and White Leghorn chicken were evaluated by assessing important parameters related to various facets of immunity such as *in-vivo* antibody response to SRBC (Fig. 4.1.1), serum lysozyme activity (Fig. 4.1.2) and serum IgG level (Fig. 4.1.3). The least squares analysis of variance, least squares means, and genetic and phenotypic parameters of the Immunocompetence (IC) traits were estimated.

### 4.1.1. Immunocompetence profiling and inheritance pattern of various immunological traits of Aseel chicken

#### 4.1.1.1 Immunocompetence (IC) status in Aseel chicken

In Aseel chicken, the results of least squares analysis of variance of IC traits and their factor-wise least square means ± S.E. are presented in Table 4.1.1.1.1 and Table 4.1.1.1.2. The least squares analysis of variance for various immunological traits revealed that the effects of sire and sex were statistically non-significant ($P > 0.05$).

The non-significant effect of sex was consistant with the reports of Sivaraman *et al.* (2005) in broiler chicken; Singh *et al.* (2010) in Aseel chicken; Gupta *et al.* (2010) in WLH chicken and Kumar *et al.* (2011) in Kadaknath chicken. These results indicated that genetic

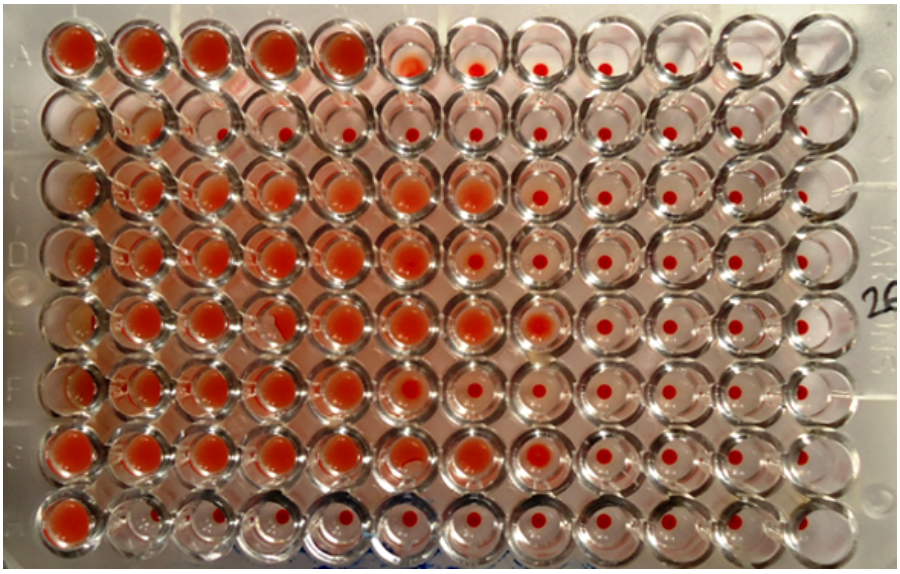


**Fig. 4.1.1 : Estimation of antibody response to SRBC using Heamagglutation test**

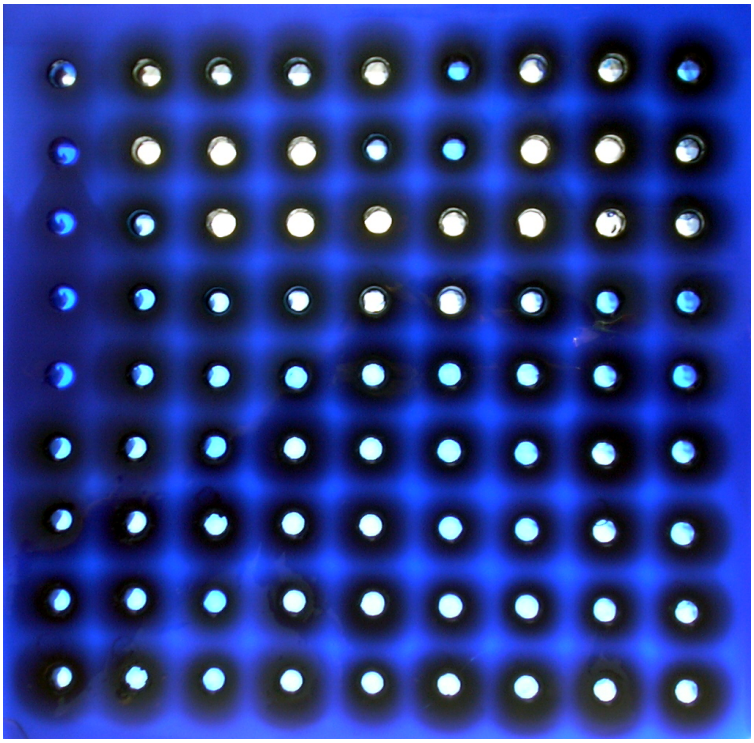


**Fig.4.1.2: Estimation of serum lysozyme concentration using Lysoplate assay**

mechanism responsible for mounting of antibody response to SRBC and regulation of serum lysozyme and serum IgG levels might be independent of sex.

**Table 4.1.1.1.1. Least squares analysis of variance of various immunocompetence traits in Aseel chicken**

| Source of variation | df | Mean sum of squares | | |
|---|---|---|---|---|
| | | HA titre | Serum Lysozyme level | Serum IgG level |
| Sire | 7 | 1.183 | 50.70 | 489.76 |
| Sex | 1 | 1.93 | 46.87 | 454.36 |
| Remainder | 64 | 3.02 | 35.93 | 793.18 |

The least squares means of HA titre, serum lysozyme and serum IgG levels 10.85 ± 0.20, 21.23 ± 0.88 µg/ml and 24.47 ± 3.32 µg/µl respectively. Females demonstrated slightly higher HA titre (11.02 ± 0.31) and IgG concentration (27.10 ± 5.08 µg/µl) but lower serum lysozyme concentration (20.38 ± 1.20 µg/ml) than males (10.68 ± 0.27, 21.83 ± 4.52 µg/µl and 22.08 ± 1 .09 µg/ml) although the differences were statistically non-significant (P > 0.05).

**Table 4.1.1.1.2. Least squares means±S.E. of various immunocompetence traits in Aseel chicken**

| Factor | HA titre | Serum Lysozyme level (µg/ml) | Serum IgG level (µg/ml) |
|---|---|---|---|
| Overall (73) | 10.85 ± 0.20 | 21.23 ± 0.88 | 24.47 ± 3.32 |
| Male (41) | 10.68 ± 0.27 | 22.08 ± 1 .09 | 21.83 ± 4.52 |
| Female(32) | 11.02 ± 0.31 | 20.38 ± 1.20 | 27.10 ± 5.08 |

*Figures within parentheses denote number of observations.*

### 4.1.1.2. Inheritance pattern of IC traits in Aseel

The heritability and correlations ($r_G$ and $r_P$) are presented in Table 4.1.1.2

The heritability estimate of serum lysozyme level was very low (0.18 ± 0.32) and not significantly difference from zero and HA titre and Serum IgG level could not be estimated. Gupta *et al.* (2010) also reported that the heritability estimates of various IC traits were very low (0.009±0.059 to 0.049±0.083) in the HSRBC line and could not be estimated in LSRBC line of White Leghorn and attributed to the low number of observation, which might be the reason in the present study as well.

**Table 4.1.1.2. Paternal half-sib heritability estimates (at diagonal), genotypic (above diagonal) and phenotypic (below diagonal) correlations among various immuno-competence traits in Aseel chicken**

| Traits | HA titre | Serum Lysozyme level | Serum IgG level |
|---|---|---|---|
| HA (65) | - | - | - |
| LZM (65) | 0.20 | 0.18 ± 0.32 | - |
| IgG (65) | 0.02 | 0.02 | - |

*Figures within parenthesis denote number of observations.*

**The genetic correlation ($r_G$) coefficients among IC traits** could not be estimated as the heritability of HA titre and serum IgG were also not estimated. Gupta *et al.* (2010) also reported that the $r_G$ was not estimable in IWJ-high SRBC line. However, Singh *et al*. (2010) reported high and positive genetic correlation ($r_G$) between serum lysozyme and serum IgG levels in Aseel chicken.

The phenotypic correlations ($r_P$) among IC traits were positive but low in magnitude (HA titre and lysozyme: 0.20; HA titre and IgG: 0.02).

Kean *et al*. (1994); Sivaraman *et al*. (2005) and Singh *et al*. (2009) also reported lower phenotypic correlations ($r_p$) among IC traits.

### 4.1.2. Immunocompetence profiling and inheritance pattern of various IC traits of Kadaknath chicken

#### 4.1.2.1 Immunocompetence (IC) status in Kadaknath chicken

In Kadaknath chicken, the results of least squares analysis of variance of IC traits and their factor-wise least square means ± S.E. are presented in Table 4.1.2.1.1 and Table 4.1.2.1.2. Least squares analysis of variance for various immunological traits revealed that the influence of sire and sex on HA titre, serum lysozyme level and serum IgG level were statistically non-significant (P > 0.05). Jaiswal *et al.* (2011) also reported non-significant effect of sex on HA titre, serum lysozyme level and serum IgG level but significant effect of sire on IC traits in Kadkanath chicken.

**Table 4.1.2.1.1. Least squares analysis of variance of various immunocompetence traits in Kadaknath chicken**

| Source of variation | df | Mean sum of squares | | |
|---|---|---|---|---|
| | | HA titre | Serum Lysozyme level | Serum IgG level |
| Sire | 7 | 4.39 | 71.20 | 44.79 |
| Sex | 1 | 2.11 | 106.72 | 24.72 |
| Remainder | 43 | 2.62 | 105.92 | 84.76 |

The least squares means of HA titre, serum lysozyme and serum IgG levels were11.69 ± 0.32, 17.13 ± 1.46 µg/ml and 16.05 ± 1.31 µg/µl respectively. The male birds have slightly higher HA titre, serum lysozyme concentration and serum IgG concentration (11.93 ± 0.45, 18.84 ± 2.46 µg/ml and 16.88 ± 2.20 µg/µl respectively) than female (11.44 ± 0.39, 15.41 ± 2.02 µg/ml and 15.23 ± 1.80 µg/µl, respectively). Gupta *et al.*

(2010), Kumar and Kumar (2011) reported non-significant influence of sex on HA titre, although males revealed higher mean antibody titre than females. Saxena *et al*. (1997), Nath (1999), Sivaraman (2004) and Singh (2005) have also reported the same in different genetic stocks. Kumar and Kumar (2011) observed higher average serum IgG concentration in males than in females.

**Table 4.1.2.1.2. Least squares means ±S.E. of various immunocompetence traits in Kadaknath chicken**

| Factor | HA titre | Serum Lysozyme level (µg/ml) | Serum IgG level (µg/ml) |
|---|---|---|---|
| Overall (52) | 11.69 ± 0.32 | 17.13 ± 1.46 | 16.05 ± 1.31 |
| Male (20) | 11.93 ± 0.45 | 18.84 ± 2.46 | 16.88 ± 2.20 |
| Female(32) | 11.44 ± 0.39 | 15.41 ± 2.02 | 15.23 ± 1.80 |

*Figures within parenthesis denote number of observations*

### 4.1.2.2. Inheritance pattern of IC traits in Kadaknath chicken

The heritability and correlations ($r_G$ and $r_p$) are presented in Table 4.1.2.2.

The heritability estimate of HA titre was moderate but not significantly different from zero (0.41 ± 0.50) and those of serum lysozyme level and serum IgG level could not be estimated. Gupta *et al.* (2010) reported very low (0.009±0.059 to 0.049±0.083) heritability estimates of various IC traits in the HSRBC line and could not estimate them in LSRBC line of White Leghorn.

The genetic correlation ($r_G$) coefficients among IC traits could not be estimated. Gupta *et al.* (2010) also could not estimate $r_G$ among IC traits in IWJ-high SRBC line of WLH chicken.

The phenotypic correlations ($r_p$) between HA titre and lysozyme was positive but low in magnitude and between HA titre and IgG concentration was close to zero.

Kean *et al*. (1994); Sivaraman *et al*. (2005) and Singh *et al*. (2009) also reported small phenotypic correlations ($r_p$) among IC traits.

**Table 4.1.2.2. Paternal half-sib heritability estimates (at diagonal), genotypic (above diagonal) and phenotypic (below diagonal) correlations among various immunocompetence traits in Kadaknath chicken**

| Traits | HA titre | Serum lysozyme level | Serum IgG level |
|---|---|---|---|
| HA (44) | 0.41±0 .50 | - | - |
| LZM (44) | 0.25 | - | - |
| IgG (44) | -0.03 | 0.002 | - |

*Figures within parenthesis denote number of observations*

### 4.1.3. Immunocompetence profiling and inheritance pattern of various immunological traits of WLH chicken

#### 4.1.3.1. Immunocompetence (IC) status in WLH chicken

In WLH chicken, the results of least squares analysis of variance of IC traits and their factor-wise least square means ± S.E. are presented in Table 4.1.3.1.1 and Table 4.1.3.1.2. The Least squares analysis of variance for various immunological traits revealed that the influence of sire and sex on HA titre, serum lysozyme level and serum IgG level was statistically non-significant (P > 0.05). These were consistent with the findings of Sivaraman *et al.* (2005) in broiler chicken, Singh *et al.* (2010) in Aseel chicken, Gupta *et al.* (2010) in WLH chicken and Kumar *et al.* (2011) in Kadaknath chicken.

The least squares means of HA titre, serum lysozyme and serum IgG levels were 11.95±0.13, 9.41 ± 0.44 µg/ml and 10.40 ±0.43 µg/µl respectively. The male bird's revealed slightly higher antibody titres against sheep RBCs (11.96±0.21), serum lysozyme level (10.14 ±0.68

µg/ml) and serum IgG level (10.57 ±0.67 µg/µl) than female(11.93±0.18, 8.68 ±0.60 µg/ml and 8.68 ±0.60 µg/µl, respectively). Gupta *et al.* (2010); Kumar and Kumar (2011) also found that the influence of sex on HA titre was statistically non-significant, although males has higher mean antibody titre than females. Saxena *et al*. (1997), Nath (1999), Sivaraman (2004) and Singh (2005) have also reported the same in different genetic stocks. Kumar and Kumar (2011) observed higher average serum IgG concentration in males than females.

**Table 4.1.3.1.1. Least squares analysis of variance of various immunocompetence traits in WLH chicken**

| Source of variation | df | Mean sum of squares | | |
|---|---|---|---|---|
| | | HA titre | Serum Lysozyme level | Serum IgG level |
| Sire | 20 | 1.59 | 16.89 | 17.61 |
| Sex | 1 | 0.02 | 43.36 | 2.27 |
| Remainder | 74 | 1.78 | 18.25 | 17.40 |

**Table 4.1.3.1.2. Least squares means±S.E. of various immuno-competence traits in WLH chicken**

| Factor | HA titre | Serum Lysozyme level (µg/ml) | Serum IgG level (µg/ml) |
|---|---|---|---|
| Overall (96) | 11.95±0.13 | 9.41 ±0.44 | 10.40 ±0.43 |
| Male (41) | 11.96±0.21 | 10.14 ±0.68 | 10.57 ±0.67 |
| Female(55) | 11.93±0.18 | 8.68 ±0.60 | 8.68 ±0.60 |

*Figures within parenthesis denote number of observations*

### 4.1.3.2. Inheritance pattern of IC traits in WLH chicken

The heritability and correlations ($r_G$ and $r_p$) are presented in Table 4.1.3.2.

The heritability estimate of HA titre and serum lisozyme level could not be estimated and serum IgG level was very low be estimated which was very low (0.01 ± 0.31). Gupta *et al.* (2010) also reported that

the heritability estimates of various IC traits were very low (0.009±0.059 to 0.049±0.083) in the HSRBC line and could not be estimated in LSRBC line of White Leghorn.

The genetic correlation ($r_G$) coefficients among IC traits could not be estimated. Gupta *et al.* (2010) also could not estimate $r_G$ among IC traits in IWJ-high SRBC line of WLH chicken.

The phenotypic correlations ($r_p$) between HA titre and serum lysozyme was positive but low in magnitude (0.12); between lysozyme and IgG concentration is very low (0.02) but among HA titre and IgG concentration is negative. The phenotypic correlations ($r_P$) among IC traits were small. Kean *et al*. (1994); Sivaraman *et al*., (2005) and Singh *et al*. (2009) also reported that the phenotypic correlations ($r_P$) among IC traits were small.

**Table 4.1.3.2. Paternal half-sib heritability estimates (at diagonal), genotypic (above diagonal) and phenotypic (below diagonal) correlations among various immuno-competence traits in WLH chicken**

| Traits | HA titre | Serum Lysozyme level | Serum IgG level |
|---|---|---|---|
| HA (75) | - | - | - |
| LZM (75) | 0.12 | - | - |
| IgG (75) | -0.12 | 0.02 | 0.01 ± 0.31 |

*Figures within parenthesis denote number of observations*

### 4.1.4 Genotypic differences in IC traits

The IC traits was pooled over three breeds i.e. Aseel, Kadaknath and WLH chicken and analysed for comparision among breed. The least square ANOVA and least square means ± S.E. are presented in Table 4.1.4.1 and Table 4.1.4.2.

High significant difference (P<0.01) among breeds for antibody titre against sheep RBCs, Serum Lysozyme level and serum IgG level

were observed. The least square means of serum lysozyme level (21.28 ± 0.78 µg/ml) and Serum IgG level (24.23 ± 2.05 µg /µl) was relatively higher in Aseel as compared to Kadaknath (16.91±0.93 and 15.70±2.44, respectively) and WLH chicken (9.42±0.68 and 10.27±1.79, respectively). However, the antibody titre against sheep RBCs was relatively lower in Aseel (10.84 ± 0.18) than the Kadaknath (11.62±0.21) and WLH chicken (11.94±0.15). Kundu *et al*. (1999) also reported that the Aseel bird had better immunocompetence than other breeds.

**Table 4.1.4.1. Least squares analysis of variance of various immunocompetence traits in Aseel, Kadaknath and WLH chicken**

| Source of variation | df | Mean sum of squares | | |
|---|---|---|---|---|
| | | HA titre | Serum Lysozyme level | Serum IgG level |
| GG | 2 | 24.93** | 2994.66** | 3989.47** |
| Sire : GG | 34 | 2.03 | 2994.66 | 119.22 |
| Sex | 1 | 0.01 | 171.00 | 119.22 |
| Remainder | 183 | 2.42 | 44.98 | 306.48 |

** *P≤0.01*

**Table 4.4.4.2: Least squares means±S.E. of various immunocompetence traits in Aseel, Kadaknath and WLH chicken**

| Factor | HA titre | Serum Lysozyme level (µg/ml) | Serum IgG level (µg/ml) |
|---|---|---|---|
| **Genotype** | | | |
| ASL | 10.84±0.18$^{b}$ | 21.28±0.78$^{a}$ | 24.23±2.05$^{a}$ |
| KAD | 11.62±0.21$^{a}$ | 16.91±0.93$^{b}$ | 15.70±2.44$^{b}$ |
| WLH | 11.94±0.15$^{a}$ | 9.42±0.68$^{c}$ | 10.27±1.79$^{c}$ |
| **Pooled Over** | | | |
| Male | 11.46±0.16 | 16.83±0.70 | 16.03±1.84 |
| Female | 11.47±.15 | 14.90±0.65 | 17.44±1.70 |

*Means within a variable with different superscripts differ significantly (P < 0.01)*

## 4.2. Objective 2: To determine immune response to ND vaccine in Aseel, Kadaknath and White Leghorn chicken

The immunity to NDV was evaluated by measuring antibody titer in the sera through haemagglutination inhibition (HI) test and Enzyme Linked Immune Sorbant Assay (ELISA) test.

### 4.2.1. Immune response to ND vaccine using HI test

Least squares analysis of variance of Antibody titre against NDV vaccine by HI test for Aseel, Kadaknath and White Leghorn chicken given in Table 4.2.1.1. The differences among anti-body titre against NDV at different days post-immunization were significant (P<0.01) except in WLH chicken.

Nasser *et al.* (2000); Kafi *et al*. (2003); Shuaib *et al.* (2003); Sasipreeyajan (2005); Jalil *et al*. (2009) and Yan *et al.* (2011) have also reported significant differences among anti-body titre against NDV at different days post-immunization for ND vaccine using HI test.

**Table 4.2.1.1. Least squares analysis of variance of Antibody titre against NDV vaccine by HI test**

| Source of variation | df | Mean sum of squares | | |
|---|---|---|---|---|
| | | Aseel | Kadaknath | WLH |
| Between dpi's | 5 | 9.90** | 32.45** | 7.92 |
| Within dpi's | 383 | 1.98 | 1.76 (432) | 7.15 (474) |
| Total | 388 | | (437) | (479) |

*Figures within parentheses denote degree of freedom*
**P<0.01

The estimated geometric mean HI titres ($log_2$) for Aseel, Kadaknath and White leghorn chicken are presented in Table 4.2.1.2.

**Table 4.2.1.2. Antibody titre against NDV vaccine by HI test**

| | 7 dpi | 14 dpi | 21 dpi | 28 dpi | 35 dpi | 42 dpi |
|---|---|---|---|---|---|---|
| Aseel | $8.10^{a}$ ±0.22 | $7.89^{a}$ ±0.18 | $7.92^{a}$ ±0.16 | $8.29^{ab}$ ±0.14 | $8.40^{ab}$ ±0.16 | $8.94^{b}$ ±0.19 |
| Kadaknath | $7.32^{a}$ ±0.16 | $7.74^{a}$ ±0.13 | $7.56^{a}$ ±0.13 | $7.86^{ab}$ ±0.17 | $8.43^{b}$ ±0.17 | $9.14^{c}$ ±0.16 |
| WLH | 8.48 ±0.28 | 8.02 ±0.31 | 8.29 ±0.33 | 8.14 ±0.30 | 7.68 ±0.29 | 7.73 ±0.29 |

*Means within a row with different superscripts differ significantly (P < 0.01)*

In Aseel native chicken, the estimated geometric mean of HI titres ($log_2$ antibody titre) were 8.10±0.22, 7.89±0.18, 7.92±0.16, 8.29±0.14, 8.40±0.16 and 8.94±0.19 on 7, 14, 21, 28, 35 and 42 dpi respectively (Table 4.2.1.2). Anti-body titre against NDV vaccine at 7 dpi exhibited some maternal response. Subsequently, from 14 dpi onwards, the trend was increasing, highest being at 42 dpi (Fig. 4.2.1). Nasser *et al.* (2000) recorded significant rise of HI antibody titre following booster dose given three weeks after the first vaccination in broiler chicks. Yan *et al.* (2011) were evaluated antibody titres of sera from post-vaccinated experimental WLH chickens by HI test. HI antibody titres to live ND vaccine gradually rose from day 7 post-vaccination and peaked at day 21.

In Kadaknath native chicken, the estimated geometric mean of HI titres ($log_2$ antibody titre) were 7.32±0.16, 7.74±0.13, 7.56±0.13, 7.86±0.17, 8.43±0.17 and 9.14±0.16 on 7, 14, 21, 28, 35 and 42 dpi respectively (Table 4.2.1.2). First peak was observed at 14 dpi, then decrease at 21 dpi and continuously increasing trend thereafter (Fig.4.2.1). Sivaraman and Kumar (2010) also reported that the NDV vaccine response peaked at 14 dpi in both high and low IC-index line for primary vaccination to NDV. For secondary response, the peak titer was observed at 42 dpi in high line. Nasser *et al.* (2000) recorded significant rise of HI antibody titre following booster dose given three weeks after the first vaccination in broiler chicks.

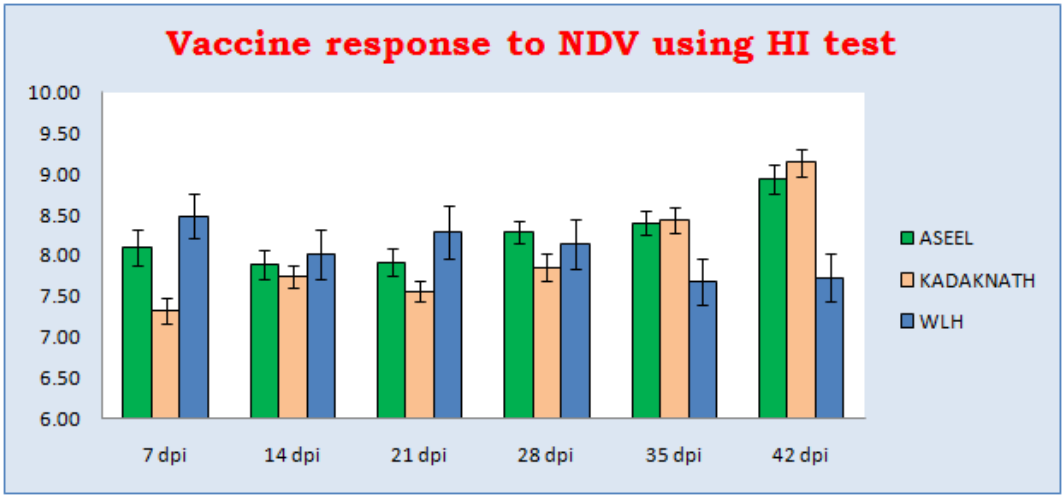



**Fig. 4.2.1. Immune response to ND vaccine using HI test.**

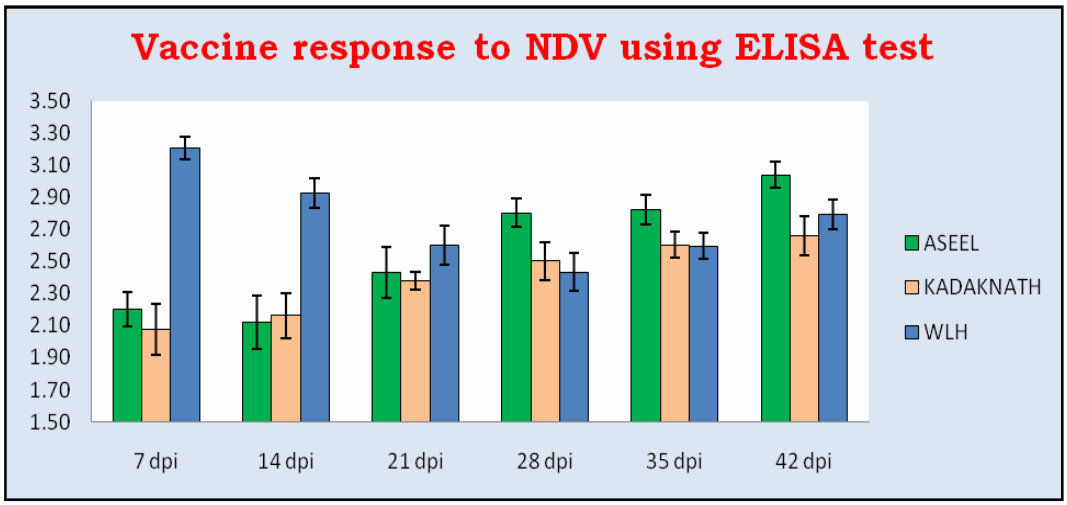



**Fig. 4.2.2. Immune response to ND vaccine using ELISA test.**

In WLH, the estimated geometric mean HI titres ($log_2$ antibody titre) were 8.48±0.28, 8.02±0.31, 8.29±0.33, 8.14±0.30, 7.68±0.29 and 7.73±0.29 on 7, 14, 21, 28, 35 and 42 dpi respectively (Table 4.2.1.2). However, higher response was observed at 7 dpi and then at 21 dpi and thereafter it revealed decreasing trend (Fig.4.2.1). But Sasipreeyajan (2005) tested serological response against NDV and found highest response at 14 dpi which declined at 21 dpi.

### 4.2.2. Immune response to ND vaccine using ELISA test

Least squares analysis of variance of antibody titre against NDV vaccine by ELISA test for Aseel, Kadaknath and White Leghorn chicken given in Table 4.2.2.1. Differences among antibody titre at different day post immunization were significant (P<0.01) in Aseel and Kadaknath chicken and also in WLH chicken at P<0.05. Sivakumar (2003), Al-Zubeedy (2009); Hauslaigner *et al.* (2009); Sivaraman and Kumar (2010) also reported significant difference among dpi by ELISA test.

**Table 4.2.2.1. Least squares analysis of variance of antibody titre against NDV vaccine by ELISA test**

| Source of variation | df | Mean sum of squares | | |
|---|---|---|---|---|
| | | Aseel | Kadaknath | WLH |
| Between dpi's | 5 | 4.59** | 1.83** | 2.70* |
| Within dpi's | 192 | 0.46 | 0.44( 182) | 0.32 (203) |
| Total | 197 | | (187) | (208) |

*Figures within parenthesis denote degree of freedom*
**P<0.01, *P<0.05

The estimated geometric mean ELISA titres ($log_{10}$ antibody titre) presented in Table 4.2.2.2.

**Table 4.2.2.2. Antibody titre against NDV vaccine by ELISA test**

| | 7 dpi | 14 dpi | 21 dpi | 28 dpi | 35 dpi | 42 dpi |
|---|---|---|---|---|---|---|
| Aseel | $2.20^{a}$ ±0.11 | $2.12^{a}$ ±0.16 | $2.43^{ab}$ ±0.16 | $2.80^{bc}$ ±0.09 | $2.82^{bc}$ ±0.09 | $3.04^{c}$ ±0.08 |
| Kadaknath | $2.08^{a}$ ±0.16 | $2.16^{ab}$ ±0.14 | $2.38^{abc}$ ±0.06 | $2.50^{abc}$ ±0.12 | $2.60^{bc}$ ±0.08 | $2.66^{c}$ ±0.12 |
| WLH | $3.20^{c}$ ±0.07 | $2.93^{bc}$ ±0.09 | $2.60^{ab}$ ±0.12 | $2.43^{a}$ ±0.12 | $2.60^{ab}$ ±0.08 | $2.79^{ab}$ ±0.09 |

*Means within a row with different superscripts differ significantly (P < 0.01)*

In Aseel, the weekly estimated geometric mean ELISA titres ($log_{10}$ antibody titre) were 2.20±0.11, 2.12±0.16, 2.43±0.16, 2.80±0.09, 2.82±0.09 and 3.04±0.08 respectively (Table 4.2.2.2). There was declining trend till 14 dpi and there after an increasing trend in antibody response was observed. The highest tire was found at 42 dpi (Fig. 4.2.2). Sivaraman and Kumar (2010) also observed highest ELISA titre against NDV vaccine at 42 dpi in high IC-index line.

In Kadaknath chicken, the weekly estimated geometric mean ELISA titres ($log_{10}$ antibody titre) were 2.08±0.16, 2.16±0.14, 2.38±0.06, 2.50±0.12, 2.60±0.08 and 2.66±0.12 respectively (Table 4.2.2.2). There was an increasing trend in the vaccine response from 7 dpi to 42 dpi (Fig.4.2.2). The highest tire was found at 42 dpi (Fig.4.2.2). Sivaraman and Kumar (2010) was also observed highest ELISA titre at 42 dpi in high IC-index line to NDV vaccine.

In WLH chicken, the estimated geometric mean ELISA titres ($log_{10}$ antibody titre) were 3.20±0.07, 2.93±0.09, 2.60±0.12, 2.43±0.12, 2.60±0.08 and 2.79±0.09 respectively (Table 4.2.2.2). There was declining trend until 28 dpi and inclining trend thereafter (Fig.4.2.2). The highest antibody titre was found at 7 dpi that revealed the vaccination of day-old chicks is very important to enhance the maternal derived antibody response. Al-Zubeedy (2009) studied antibody titers in one day old

broiler chicks from four groups of broiler breeder chickens using ELISA test and revealed that the vaccination of day-old chicks is very important to enhance the maternal derived antibody response.

### 4.2.3. Comparison between HI titres and ELISA titres

#### 4.2.3.1. Immune response to ND vaccine in Aseel chicken

In Aseel native chicken, the estimated weekly geometric mean HI titres ($log_2$ antibody titre) and mean ELISA titres ($log_{10}$ antibody titres) have been given in Table 4.2.1.2 and Table 4.2.2.2 respectively. Differences among dpi's were significant (P<0.01) for HI titres and ELISA titres. Both having declined trend till 14 dpi, subsequently from 14 dpi onwards, the trend was increasing, highest being at 42 dpi (Fig. 4.2.3.1). Consistent pattern in the amtibody levels by haemagglutination inhibition (HI) test and enzyme-linked immunosorbent assay (ELISA) were observed. Hauslaigner *et al.* (2009) also observed a positive linear correlation between both tests in Muscovy ducks (*Cairina moschata*) and domestic geese (*Anser anser* var. *domestica*). However, HI was confirmed more cheaper than ELISA as no micro plate reader is required in addition to the cost of ELISA kit. Similar finding was also published by Bozorghmehrifardl and Mayahi, (2000) who showed that HI test is more economic than ELISA kit to be used in used for detection of antibody levels against NDV.

#### 4.2.3.2. Immune response to ND vaccine in Kadaknath chicken

The weekly mean HI titres ($log_2$ antibody titre) and ELISA titres ($log_{10}$ antibody titre) in Kadaknath native chicken have been presented in Table 4.2.1.2 and 4.2.2.2 respectively. Differences among dpi for HI titres and ELISA titres were significant (P<0.01). The trend was non-consistent between the two tests. Tabidi *et al.* (2004) compared the antibody (Ab) titres to the intermediate Newcastle disease virus (NDV) vaccine (Komorov strain) in broiler chicks using haemagglutination

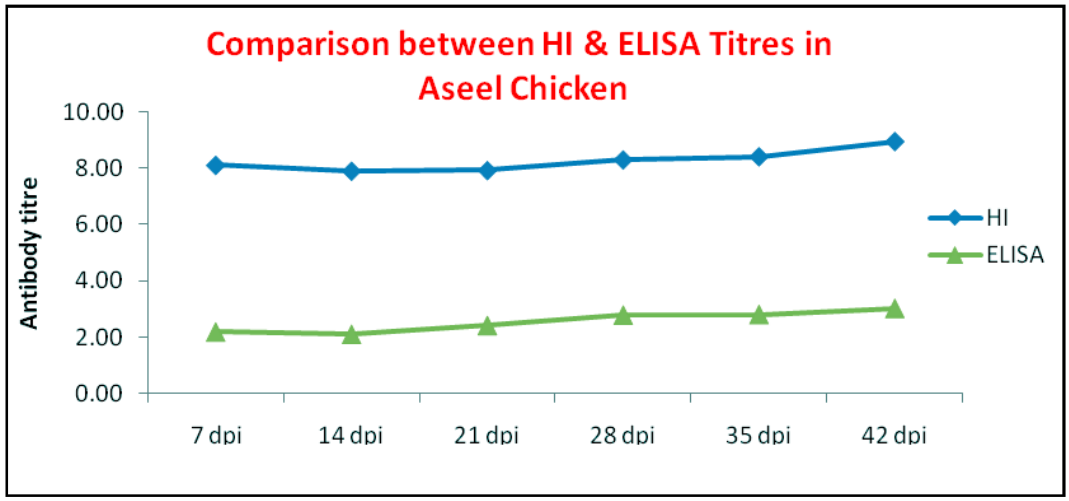



**Fig. 4.2.3.1. Immune response to ND vaccine in ASEEL chicken**

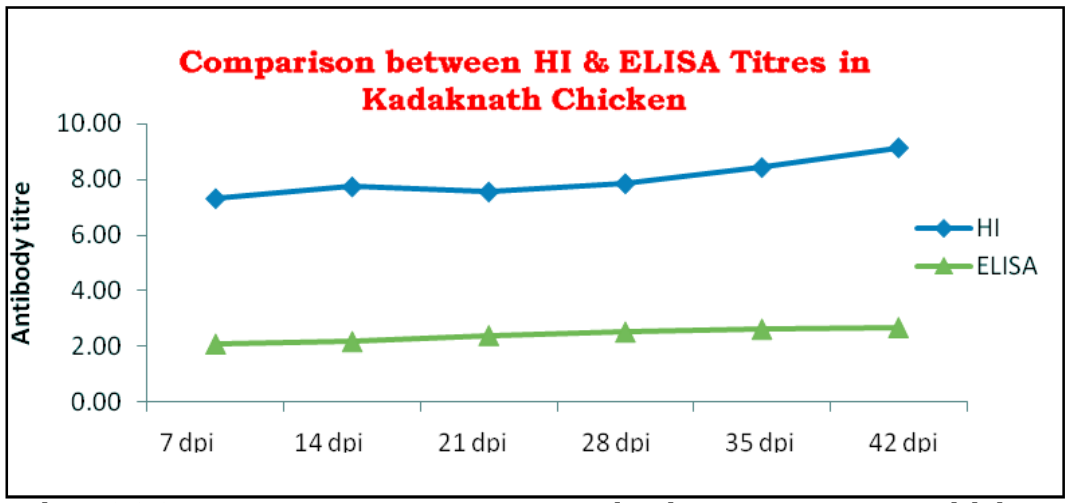



**Fig. 4.2.3.2. Immune response to ND vaccine in KADAKNATH chicken**

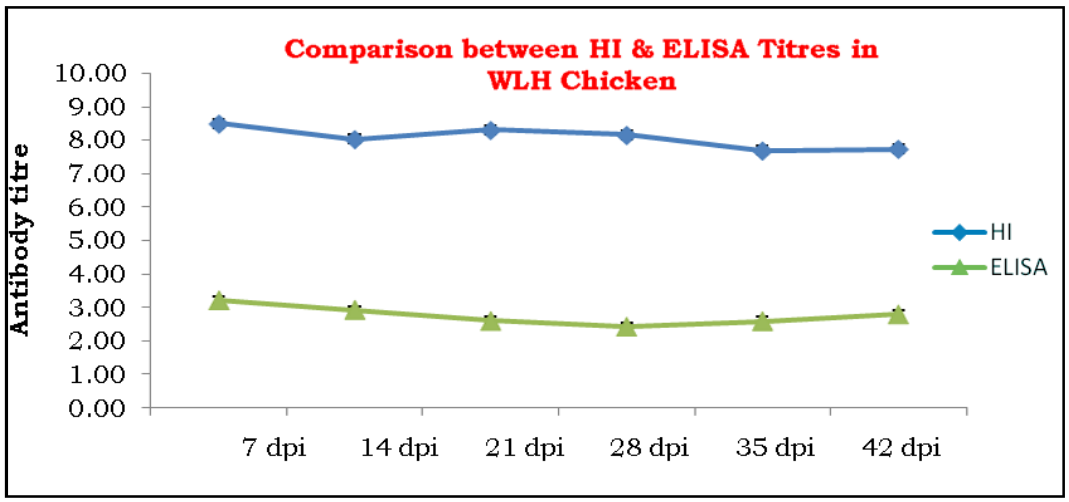



**Fig. 4.2.3.3 Immune response to ND vaccine in WLH chicken**

inhibition (HI) test and an indirect enzyme-linked immunosorbent assay (ELISA) and reported non-consistent pattern in the antibody titres estimated by two tests.

#### 4.2.3.3. Immune response to ND vaccine in WLH chicken

The weekly mean HI titres ($log_2$ antibody titre) and ELISA titres ($log_{10}$ antibody titre) In WLH chicken have been presented in Table 4.2.1.2 and 4.2.2.2 respectively. Differences among dpi's were non-significant (P>0.05) for HI test but significant (P<0.05) for ELISA test (Table 4.2.3.3.1). There was non-consistent pattern in the antibody titres between HI test and ELISA test (Fig. 4.2.3.3). Tabidi *et al.* (2004) also observed the non-consistent pattern in the antibody levels between two tests in broilers chicken (Komorov strain).

### 4.2.4. Comparison among genotypes in vaccine response to NDV vaccine

#### 4.2.4.1 Using HI test

The estimated geometric mean HI titres ($log_2$) for Aseel, Kadaknath and White Leghorn chicken are presented in Table 4.2.1.1.

**Table 4.2.4.1. HI titres against NDV vaccine among different genotypes**

| | Aseel | Kadaknath | WLH |
|---|---|---|---|
| 7 dpi | 8.10±0.22$^{a}$ | 7.32±0.16$^{b}$ | 8.48±0.28$^{a}$ |
| 14 dpi | 7.89±0.18$^{a}$ | 7.74±0.13$^{a}$ | 8.02±0.31$^{a}$ |
| 21 dpi | 7.92±0.16$^{a}$ | 7.56±0.13$^{a}$ | 8.29±0.33$^{a}$ |
| 28 dpi | 8.29±0.14$^{a}$ | 7.86±0.17$^{a}$ | 8.14±0.30$^{a}$ |
| 35 dpi | 8.40±0.16$^{ab}$ | 8.43±0.17$^{a}$ | 7.68±0.29$^{b}$ |
| 42 dpi | 8.94±0.19$^{a}$ | 9.14±0.16$^{a}$ | 7.73±0.29$^{b}$ |

*Means within a row with different superscripts differ significantly (P < 0.01)*

Genotype differences in vaccine response to NDV vaccine using HI test observed significant (P<0.05) at 7 dpi, 35 dpi and 42 dpi only. At

7 dpi WLH chicken showing highest antibody titre (8.48±0.28) following Aseel and Kadaknath chicken (8.10±0.22 and 7.32±0.16, respectively) while at 35 and 42 dpi Kadaknath chicken (8.43±0.17 and 9.14±0.16, respectively) showing highest titre followed by Aseel (8.40±0.16 and 8.94±0.19, respectively) and WLH chicken (7.68±0.29 and 7.73±0.29, respectively).

#### 4.2.4.2 Using ELISA test

The estimated geometric mean ELISA titres ($log_{10}$) for Aseel, Kadaknath and White Leghorn chicken are presented in Table 4.2.4.2.

**Table 4.2.4.2. ELISA titres against NDV vaccine among different genotypes**

| | Aseel | Kadaknath | WLH |
|---|---|---|---|
| 7 dpi | 2.20±0.11$^{b}$ | 2.08±0.16$^{b}$ | 3.20±0.07$^{a}$ |
| 14 dpi | 2.12±0.16$^{b}$ | 2.16±0.14$^{b}$ | 2.93±0.09$^{a}$ |
| 21 dpi | 2.43±0.16$^{a}$ | 2.38±0.06$^{a}$ | 2.60±0.12$^{a}$ |
| 28 dpi | 2.80±0.09$^{a}$ | 2.50±0.12$^{a}$ | 2.43±0.12$^{a}$ |
| 35 dpi | 2.82±0.09$^{a}$ | 2.60±0.08$^{a}$ | 2.60±0.08$^{a}$ |
| 42 dpi | 3.04±0.08$^{a}$ | 2.66±0.12$^{b}$ | 2.79±0.09$^{ab}$ |

*Means within a row with different superscripts differ significantly (P < 0.01)*

Genotype differences in vaccine response to NDV vaccine using ELISA test observed significant (P<0.05) at 7 dpi, 14 dpi and 42 dpi only. At 7 dpi WLH chicken showing highest antibody titre (3.20±0.07) followed by Aseel (2.20±0.11) and Kadaknath chicken (2.08±0.16) while at 14 dpi WLH chicken showing highest antibody titre (2.93±0.09) followed by Kadaknath chicken (2.16±0.14) and Aseel chicken (2.12±0.16). At 42 dpi Aseel chicken (3.04 ± 0.08) showing highest titre followed by WLH chicken (2.79±0.09) and Aseel chicken (2.66±0.12).

## 4.3. Objective 3: To study the relative expression of few important immunity related candidate genes in various tissues viz. spleen, thymus and bursa of Aseel, Kadaknath and White Leghorn chicken by quantitative reverse transcription PCR (qRT-PCR)

The mRNA expression of four immunity related genes were studied in three tissues (bursa, spleen and thymus) collected from three genotypes (Aseel, Kadaknath and WLH) and the data were analyzed, firstly to assess differences in expression levels among genotypes for four genes in a particular tissue. Secondly, the data was analyzed to assess the differences in expression levels among tissues in three genotypes for each of the four genes. Thirdly, the data was analyzed to assess differences in expression levels among genes in three tissues in a particular genotype.

### 4.3.1. Genotypic differences among Aseel, Kadaknath and White Leghorn chicken for mRNA expression of immune related genes in different tissues

The data on mRNA expression of four immunity related genes was analyzed to assess differences in expression levels among genotypes for four genes in a particular tissue. The results are presented in Tables 4.3.1.1.2, 4.3.1.2.1, 4.3.1.2.2, 4.3.1.3.1 and 4.3.1.1.2

#### *4.3.1.1. Expression in bursa*

The LS ANOVA for assessing the influence of genotype and sex on the mRNA expression of four immunity related genes (IL1-beta, IFN-$\gamma$, iNOS and TLR15) in the bursa is presented in the table 4.3.1.1.1. The least square means ± S.E. of the adjusted mean Ct values are presented in Table 4.3.1.1.2.

**Table 4.3.1.1.1 Effects of genotype and sex on mRNA expression of immune-related genes (P values, ANOVA) in the bursa of Aseel, Kadaknath and WLH chicks**

| Source of Variation | df | P Values for specific mRNA gene expression | | | |
|---|---|---|---|---|---|
| | | Genes | | | |
| | | IL1-β | IFN-γ | iNOS | TLR15 |
| Genotypes | 2 | 0.112 | 0.495 | 0.353 | 0.321 |
| Sex | 1 | 0.342 | 0.917 | 0.924 | 0.908 |

**Table 4.3.1.1.2 Factor-wise least squares means of adjusted Ct values of mRNA expression of immune-related genes in bursa of Aseel, Kadaknath and WLH chicks**

| Factor (s) | LS mean of adjusted Ct values of mRNA expression levels | | | |
|---|---|---|---|---|
| | Genes | | | |
| | IL1-β | IFN-γ | iNOS | TLR15 |
| **Genotypes** | | | | |
| Aseel | 11.40 ± 1.22 | 14.21 ± 0.52 | 12.56 ± 1.35 | 11.73 ± 0.95 |
| Kadaknath | 15.06 ± 1.04 | 13.44 ± 0.52 | 15.48 ± 1.35 | 13.64 ± 0.95 |
| WLH | 12.26 ± 1.04 | 13.39 ± 0.52 | 13.59 ± 1.35 | 11.77 ± 0.95 |
| **Sex** | | | | |
| Male | 12.26 ± 0.95 | 13.71 ± 0.42 | 13.96 ± 1.10 | 12.31 ± 0.77 |
| Female | 13.56 ± 0.85 | 13.65 ± 0.42 | 13.80 ± 1.10 | 12.44 ± 0.77 |

It was observed that neither genotype nor sex had significant ($p > 0.05$) effect on the expression of any of the four genes studied. However, genotype showed little influence ($p=0.112$) on the mRNA expression of IL1-beta in bursa tissue.

### 4.3.1.2. Expression in Spleen

The LS ANOVA for assessing the influence of genotype and sex on the mRNA expression of four immunity related genes in the spleen

is presented in the Table 4.3.1.2.1. The least square means ± S.E. of the adjusted Ct values are presented in Table 4.3.1.2.2.

**Table 4.3.1.2.1: Effects of genotypes and sex on mRNA expression of immune-related genes (P values, ANOVA) in spleen of Aseel, Kadaknath and WLH chicken**

| Source of Variation | df | P Values for specific mRNA gene expression | | | |
|---|---|---|---|---|---|
| | | Genes | | | |
| | | IL1-β | IFN-γ | iNOS | TLR15 |
| Genotypes | 2 | 0.138 | 0.379 | 0.011* | 0.096 |
| Sex | 1 | 0.875 | 0.504 | 0.142 | 0.574 |

*P<0.01

The genotypic differences among Aseel, Kadaknath and White Leghorn chicken were significant (p<0.05) for mRNA expression of iNOS gene and to some extent on TLR15 (P=0.096). Sex effect was non-significant (P > 0.05) on mRNA expression of all the four genes studied.

**Table 4.3.1.2.2. Factor-wise least squares means of adjusted Ct values of mRNA expression of immune-related genes in spleen of Aseel, Kadaknath and WLH chicken**

| Factor (s) | LS mean of adjusted Ct values of mRNA expression levels | | | |
|---|---|---|---|---|
| | Genes | | | |
| | IL1-β | IFN-γ | iNOS | TLR15 |
| **Genotypes** | | | | |
| Aseel | 23.76±0.49 | 29.34±0.09 | -2.26±1.25$^{b}$ | 26.90±0.32 |
| Kadaknath | 25.05 ± 0.42 | 29.48±0.09 | 4.93±1.25$^{a}$ | 27.68±0.32 |
| WLH | 23.87 ± 0.42 | 29.52±0.09 | 0.97±1.25$^{ab}$ | 26.54±0.32 |
| **Sex** | | | | |
| Male | 24.27 ± 0.38 | 29.41 ± 0.07 | 0.03 ± 1.02 | 26.93 ± 0.26 |
| Female | 24.18 ± 0.34 | 29.48 ± 0.07 | 2.39 ± 1.02 | 27.15 ± 0.26 |

*Means within a factor in a column with different superscripts differ significantly (P < 0.05)*

The non-significant difference in the mRNA expression of IL1-beta observed in the present study was in line with the finding of Cheeseman *et al.* (2007) who also observed non-significant effect of breed on the pro-inflammatory cytokines like IL1-β in spleen while analyzing the three distinct breeds viz., broiler, Fayoumi and Leghorn.

The mRNA expression of iNOS gene in spleen tissue was highest in Kadaknath chicken followed by WLH and Aseel chicken. The genotypic differences were in consonance with the report of Aricibasi (2010).

Aricibasi (2010) however, reported that the regulation of IFN-γ mRNA expression in the spleen was significantly affected by genetic background, but in the present study the difference among genotypes were non-significant. Further study may provide deep insight in this issue.

### *4.3.1.3. Expression in thymus*

The LS ANOVA for assessing the influence of genotype and sex on the mRNA expression of four immunity related genes in thymus tissue is presented in table 4.3.1.3.1. The least square means ± S.E. of the adjusted Ct values are presented in table 4.3.1.1.2.

**Table *4.3.1.3*.1: Effects of genotype and sex on mRNA expression of immune-related genes (P values, ANOVA) in thymus of Aseel, Kadaknath and WLH chicken**

| Source of Variation | df | P Values for specific mRNA gene expression | | | |
|---|---|---|---|---|---|
| | | Genes | | | |
| | | IL1-β | IFN-γ | iNOS | TLR15 |
| Genotypes | 2 | 0.008** | 0.174 | 0.066 | 0.070 |
| Sex | 1 | 0.298 | 0.957 | 0.200 | 0.013* |

*P<0.05; **P<0.01

Analysis revealed that genotypic differences for mRNA expression of IL1-β gene among Aseel, Kadaknath and White Leghorn chicken were significant ($p \leq 0.01$). Kadaknath chicken showed highest mRNA expression of IL1-β gene followed by WLH and Aseel chicken. Kumar *et al*. (2011) however reported that the mRNA expression of IL1-beta, iNOS and TLR 15 did not differ significantly among genetic lines, viz., broiler, Fayoumi and WLH.

The present finding of non-significant differences in TLR15 mRNA expression among genotypes is in consonance with the report of Nerren *et al*. (2009) who observed that the expression of TLR15 varies in heterophils from different chicken lines in response to stimulation with Salmonella *enterica serovar* Enteritidis, but not in the non-stimulated cells.

**Table 4.3.1.3.2: Factor-wise least squares means of adjusted Ct values of mRNA expression of immune-related genes in thymus of Aseel, Kadaknath and WLH chicken**

| Items | LS mean of adjusted Ct values of mRNA expression levels | | | |
|---|---|---|---|---|
| | Genes | | | |
| | IL1-β | IFN-γ | iNOS | TLR15 |
| **Genotypes** | | | | |
| Aseel | 12.34 ± 0.91[b] | 31.06 ± 0.10 | 24.61 ± 0.65 | 11.07 ± 0.54 |
| Kadaknath | 17.45 ± 0.78[a] | 31.14 ± 0.08 | 26.73 ± 0.65 | 11.56 ± 0.54 |
| WLH | 13.63 ± 0.78[b] | 31.33 ± 0.08 | 26.97 ± 0.65 | 9.52 ± 0.54 |
| **Sex** | | | | |
| Male | 15.01 ± 0.71 | 31.19 ± 0.07 | 25.57 ± 0.53 | 11.72 ± 0.44[a] |
| Female | 13.94 ± 0.63 | 31.18 ± 0.07 | 26.63 ± 0.53 | 9.71 ± 0.44[b] |

*Means within a factor in a column with different superscripts differ significantly (P < 0.05)*

The effect of sex was significant ($p<0.05$) only on TLR15 mRNA expression, but not on other three genes (IL1-beta, IFN-$\gamma$, and iNOS). TLR15 mRNA expression was higher in male than female.

Contrary to the observation in the present finding, Kumar *et al.* (2011) reported that the expression of IL1-$\beta$ was significantly ($P < 0.05$) affected by sex and males exhibited higher ($P < 0.05$) IL1-$\beta$ expression than females. Contrarily for IFN-$\gamma$ also, Aricibasi (2010) reported that the regulation of IFN-$\gamma$ mRNA expression in the spleen was significantly affected by genetic background. Dil and Khureshi (2003) reported that the expression of iNOS gene in chicken varies with genetic background. Ahmad *et al.* (2007) postulated that the expression of immunity related genes like iNOS are under the control of genetic contribution.

Present findings were in agreement with those of earlier reports in some cases but were in contrast in some other cases, hence are suggestive of further study.

### 4.3.2. Tissue differences in mRNA expression of various immune related genes in Aseel, Kadaknath and White Leghorn chicken

The data on mRNA expression of four immunity related genes was analyzed to assess the differences in expression levels among tissues in three genotypes for each of the four genes and results are presented in tables 4.3.2.1.1, 4.3.2.1.2, 4.3.2.2.1, 4.3.2.2.2, 4.3.2.3.1, 4.3.2.3.2, 4.3.2.4.1 and 4.3.2.4.2. Apmplification and dissociation curve of all studied genes during qRT PRC in various tissue of Aseel, Kadaknath and White Leghorn chiceken presented in (Fig. 4.3.2.1, 4.3.2.2, 4.3.2.3, 4.3.2.4).

Having presented the results for each of the four genes, they have been discussed in light of the related published literature.

#### 4.3.2.1 In mRNA expression of IL1-Beta

The LS ANOVA for assessing the influence of tissue and sex on the mRNA expression of IL1-beta in Aseel, Kadaknath and White Leghorn chicken are presented in the Table 4.3.2.1.1. The least square means ± S.E. of the adjusted Ct values are presented in Table 4.3.2.1.2.

**Table 4.3.2.1.1 Tissue differences in mRNA expression of IL1-β gene (P values, ANOVA) in Aseel, Kadaknath and white Leghorn chicken**

| Source of Variation | df | P Values for specific mRNA gene expression | | |
|---|---|---|---|---|
| | | Genotypes | | |
| | | Aseel | Kadaknath | WLH |
| Sex | 1 | 0.6802 | 0.1507 | 0.2268 |
| Tissue | 2 | <.0001** | <.0001** | <.0001** |

**P<0.01

**Table 4.3.2.1.2 Factor-wise least squares means of adjusted Ct values of mRNA expression of IL1-β gene in Aseel, Kadaknath and white Leghorn chicken**

| Items | LS mean of adjusted Ct values of mRNA expression levels | | |
|---|---|---|---|
| | Genotypes | | |
| | Aseel | Kadaknath | WLH |
| **Sex** | | | |
| Male | 16.07 ± 0.63 | 18.42 ± 0.69 | 17.18 ± 0.64 |
| Female | 15.73 ± 0.44 | 19.97 ± 0.69 | 15.99 ± 0.64 |
| **Tissue** | | | |
| Bursa | 11.68 ± 0.64$^{b}$ | 15.07 ± 0.84$^{b}$ | 12.26 ± 0.79$^{b}$ |
| Spleen | 23.80 ± 0.64$^{a}$ | 25.06 ± 0.84$^{a}$ | 23.87 ± 0.79$^{a}$ |
| Thymus | 12.22 ± 0.64$^{b}$ | 17.46 ± 0.84$^{b}$ | 13.63 ± 0.79$^{b}$ |

*Means within a factor in a column with different superscripts differ significantly (P < 0.05)*

The mRNA expression of IL1-β varied significantly (p<0.01) among tissues in Aseel, Kadaknath and White Leghorn chicken. However, the expression did not differ (p<0.05) between sexes. The mRNA expression

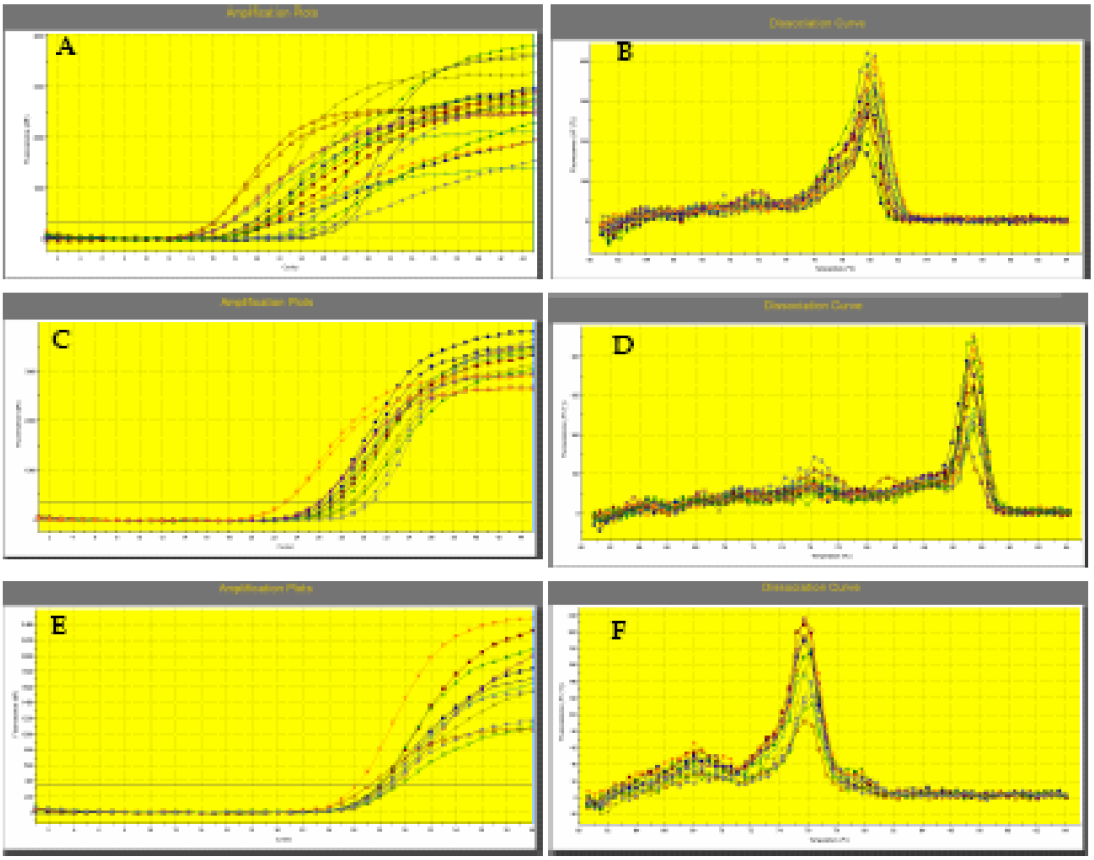



Fig. 4.3.2.1 : Amplification (A,C,E) and dissociation (B,D,F) curves of IL1- β gene mRNA during qRT-PCR in various tissues of Aseel, Kadaknath and WLH chicken (A, B: Bursa; C, D: Spleen; E, F: Thymus)

of IL1-β was highest in spleen followed by thymus and bursa in Aseel, Kadaknath and White Leghorn chicken.

#### 4.3.2.2 In mRNA expression of IFN-γ

The least squares analysis of variance of mRNA expression of IFN-γ in Aseel, Kadaknath and white Leghorn chicken are presented in the Table 4.3.2.2.1. The least square means ± S.E. of the adjusted Ct values are presented in Table 4.3.2.2.2.

**Table 4.3.2.2.1 Tissue differences in mRNA expression of IFN-γ gene (P values, ANOVA) in Aseel, Kadaknath and White Leghorn chicken**

| Source of Variation | df | P Values for specific mRNA gene expression | | |
|---|---|---|---|---|
| | | Genotypes | | |
| | | Aseel | Kadaknath | WLH |
| Sex | 1 | 0.6744 | 0.6114 | 0.1729 |
| Tissue | 2 | <0.0001** | <0.0001** | <0.0001** |

**P<0.01

**Table 4.3.2.2.2 Factor-wise least squares means of adjusted Ct values of mRNA expression of IFN-γ gene in Aseel, Kadaknath and White Leghorn chicken**

| Items | LS mean of adjusted Ct values of mRNA expression levels | | |
|---|---|---|---|
| | Genotypes | | |
| | Aseel | Kadaknath | WLH |
| **Sex** | | | |
| Male | 24.75 ± 0.39 | 24.61 ± 0.21 | 24.93 ± 0.16 |
| Female | 24.98 ± 0.35 | 24.77 ± 0.21 | 24.57 ± 0.16 |
| **Tissue** | | | |
| Bursa | 14.21 ± 0.43$^{b}$ | 13.45 ± 0.26$^{c}$ | 13.39 ± 0.20$^{c}$ |
| Spleen | 29.34 ± 0.43$^{a}$ | 29.48 ± 0.26$^{b}$ | 29.52 ± 0.20$^{b}$ |
| Thymus | 31.03 ± 0.50$^{a}$ | 31.15 ± 0.26$^{a}$ | 31.33 ± 0.20$^{a}$ |

*Means within a factor in a column with different superscripts differ significantly (P < 0.05)*

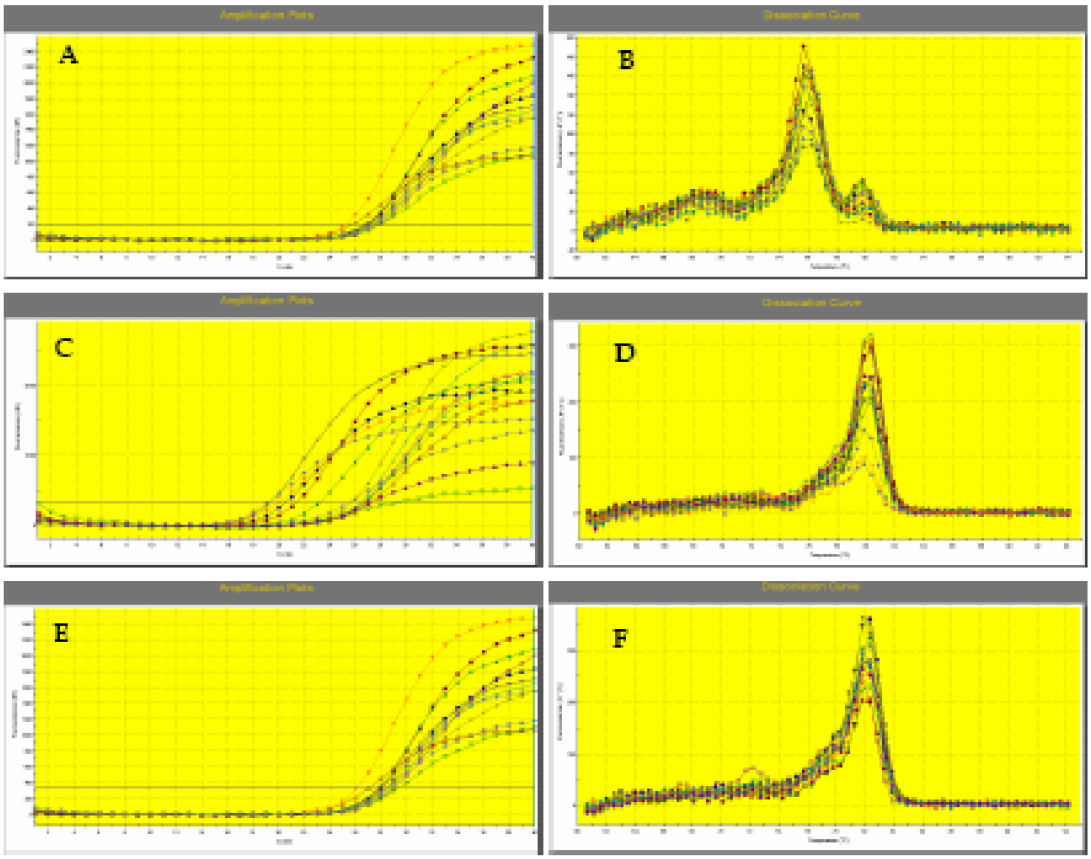



Fig. 4.3.2.2 : Amplification (A,C,E) and dissociation (B,D,F) curves of INF-γ gene mRNA during qRT-PCR in various tissues of Aseel, Kadaknath and WLH chicken (A, B: Bursa; C, D: Spleen; E, F: Thymus)

Tissue differences in mRNA expression of IFN-γ were significant in Aseel, Kadaknath and White Leghorn chicken but not the sex difference. The mRNA expression of IFN-γ was highest in thymus followed by spleen and bursa in Aseel, Kadaknath and White Leghorn chicken.

### 4.3.3.1 In mRNA expression of iNOS

The influence of tissue and sex on the mRNA expression of iNOS in Aseel, Kadaknath and White Leghorn chicken are presented in the Table 4.3.2.3.1. The least square means ± S.E. of the adjusted Ct values are presented in Table 4.3.2.3.2.

**Table 4.3.2.3.1 Tissue differences in mRNA expression of iNOS gene (P values, ANOVA) in Aseel, Kadaknath and White Leghorn chicken**

| Source of Variation | df | P Values for specific mRNA gene expression | | |
|---|---|---|---|---|
| | | Genotypes | | |
| | | Aseel | Kadaknath | WLH |
| Sex | 1 | 0.6896 | 0.5525 | 0.2055 |
| Tissue | 2 | <0.0001** | <0.0001** | <0.0001** |

**P<0.01

Differences among tissues for iNOS mRNA expression levels in Aseel, Kadaknath and white Leghorn chicken were significant, but not between sexes.

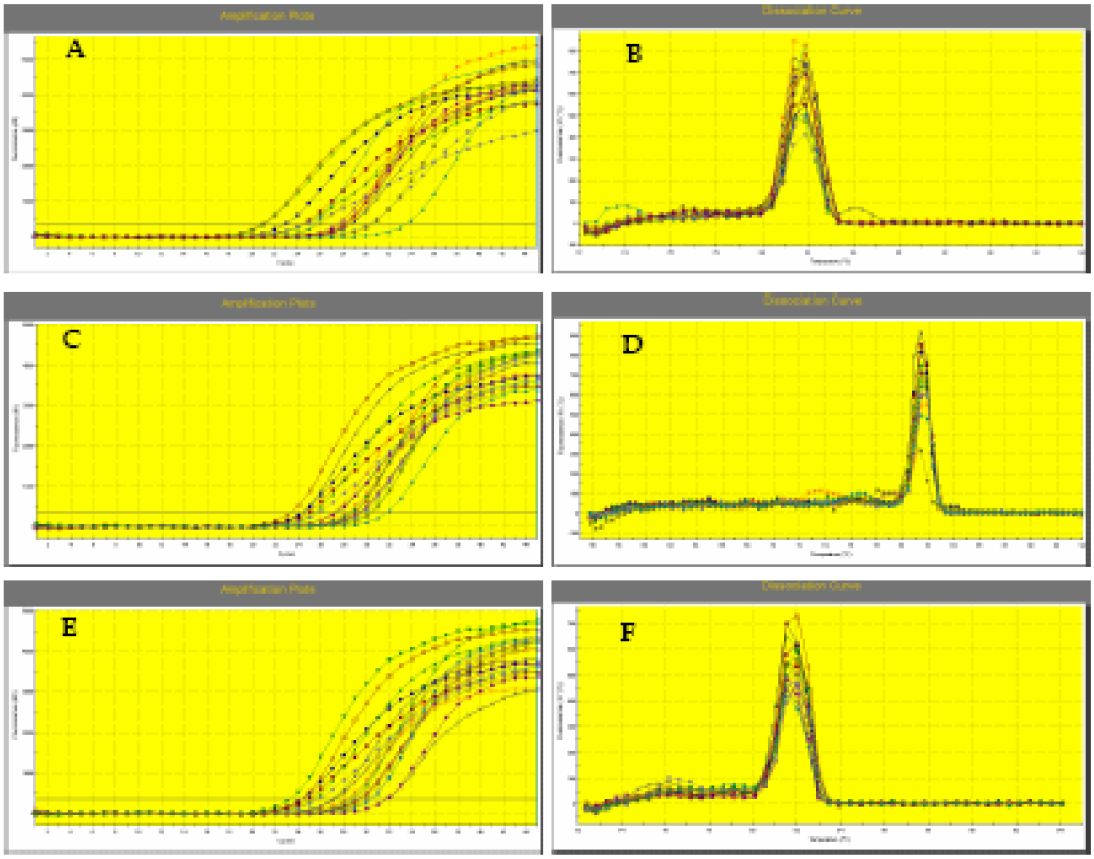



Fig. 4.3.2.3 : Amplification (A,C,E) and dissociation (B,D,F) curves of iNOS gene mRNA during qRT-PCR in various tissues of Aseel, Kadaknath and WLH chicken (A, B: Bursa; C, D: Spleen; E, F: Thymus)

**Table 4.3.2.3.2 Factor-wise least squares means of adjusted Ct values of mRNA expression of iNOS gene in Aseel, Kadaknath and White Leghorn chicken**

| Items | LS mean of adjusted Ct values of mRNA expression levels | | |
|---|---|---|---|
| | Genotypes | | |
| | Aseel | Kadaknath | WLH |
| **Sex** | | | |
| Male | 11.35 ± 0.10 | 15.35 ± 0.83 | 12.87 ± 0.99 |
| Female | 11.93 ± 0.14 | 16.08 ± 0.83 | 14.81 ± 0.99 |
| **Tissue** | | | |
| Bursa | 12.57 ± 1.22$^{b}$ | 15.48 ± 1.02$^{b}$ | 13.59 ± 1.22$^{b}$ |
| Spleen | -2.26 ± 1.22$^{c}$ | 4.93 ± 1.02$^{c}$ | 0.97 ± 1.22$^{c}$ |
| Thymus | 24.61 ±1.22$^{a}$ | 26.73 ± 1.02$^{a}$ | 26.97 ± 1.22$^{a}$ |

*Means within a factor in a column with different superscripts differ significantly (P < 0.05)*

The mRNA expression of iNOS was highest in thymus followed by bursa and spleen in Aseel, Kadaknath and White Leghorn chicken.

### 4.3.3.1 In mRNA expression of TLR15

The influence of tissue and sex on the mRNA expression of TLR15 in Aseel, Kadaknath and White Leghorn chicken are presented in the Table 4.3.2.4.1. The least square means ± S.E. of the adjusted Ct values are presented in Table 4.3.2.4.2.

**Table 4.3.2.4.1 Tissue differences in mRNA expression of TLR15 gene (P values, ANOVA) in Aseel, Kadaknath and White Leghorn chicken**

| Source of Variation | df | P Values for specific mRNA gene expression | | |
|---|---|---|---|---|
| | | Genotypes | | |
| | | Aseel | Kadaknath | WLH |
| Sex | 1 | 0.5739 | 0.5232 | 0.4998 |
| Tissue | 2 | <0.0001** | <0.0001** | <0.0001** |

**P<0.01

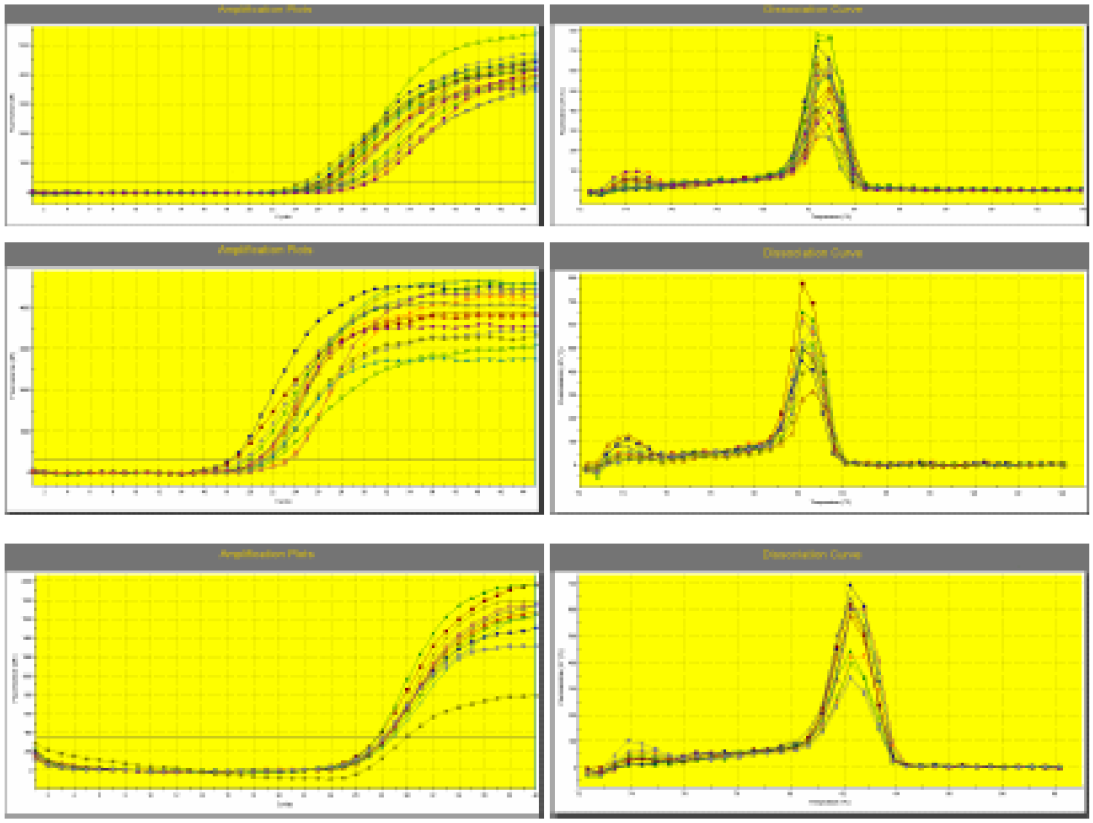


Fig. 4.3.2.4 : Amplification (A,C,E) and dissociation (B,D,F) curves of TLR 15 gene mRNA during qRT-PCR in various tissues of Aseel, Kadaknath and WLH chicken (A, B: Bursa; C, D: Spleen; E, F: Thymus)

**Table 4.3.2.4.2 Factor-wise least squares means of adjusted Ct values of mRNA expression of TLR15 gene in Aseel, Kadaknath and White Leghorn chicken**

| Items | LS mean of adjusted Ct values of mRNA expression levels | | |
|---|---|---|---|
| | Genotypes | | |
| | Aseel | Kadaknath | WLH |
| **Sex** | | | |
| Male | 16.78 ± 0.50 | 17.90± 0.57 | 16.29 ± 0.69 |
| Female | 16.36 ± 0.50 | 17.35 ± 0.57 | 15.59 ± 0.69 |
| **Tissue** | | | |
| Bursa | 11.73 ± 0.62$^{b}$ | 13.64 ± 0.70$^{b}$ | 11.77 ± 0.85$^{b}$ |
| Spleen | 26.91 ± 0.62$^{a}$ | 27.68 ± 0.70$^{a}$ | 26.54 ± 0.85$^{a}$ |
| Thymus | 11.07 ± 0.62$^{b}$ | 11.56 ± 0.70$^{b}$ | 9.52 ± 0.85$^{b}$ |

*Means within a factor in a column with different superscripts differ significantly (P < 0.05)*

Tissue had significant effect on mRNA expression of TLR15 in Aseel, Kadaknath and White Leghorn chicken but sex had non-significant influence. The mRNA expression of TLR15 was highest in spleen followed by bursa and thymus in Aseel, Kadaknath and White Leghorn chicken.

Contrarily, Higgs *et al*. (2006) observed higher expression of TLR 15 mRNA in bursa followed by spleen which might be due to different genetic backgrounds in two experimental units.

Non-significant differences in the expression of studied four genes among genotypes in almost all cases might be due the same reason as reported by Kumar *et al.* (2011) for assayed genes (IL1-beta, iNOS and TLR15) that there was lack of significant differential expression among genotypes might be an indication of an immune system that had returned to homeostasis by the time that samples were collected.

### 4.3.3 Expression differences among (IL1-β, IFN-γ, iNOS and TLR15) genes in different tissues of Aseel, Kadaknath and White Leghorn chicken

The data on mRNA expression of four immunity related genes was analyzed to assess the differences in expression levels among genes

in three tissues in a particular genotype and results are presented in Tables 4.3.2.1.1, 4.3.2.1.2, 4.3.2.2.1, 4.3.2.2.2, 4.3.2.3.1 and 4.3.2.3.2.

Having presented the results in each of the three genotypes, they have been discussed in light of the related published literature.

### 4.3.2.1 Aseel

Expression differences among genes (IL1-β, IFN-γ, iNOS and TLR15) in different tissues of Aseel chicken are presented in Table 4.3.2.1.1 and their least squares means ± SE of adjusted cycle threshold values are presented in Table 4.3.2.1.2.

**Table 4.3.2.1.1 Expression differences among IL1-β, IFN-γ, iNOS and TLR15 genes (P values, ANOVA) in different tissues of Aseel chicken**

| Source of Variation | df | P Values for specific mRNA gene expression | | |
|---|---|---|---|---|
| | | Tissue | | |
| | | Bursa | Spleen | Thymus |
| **Genes** | 3 | 0.4537 | <0.0001** | <0.0001** |
| **Sex** | 1 | 0.9308 | 0.4674 | 0.4978 |

**P<0.01

**Table 4.3.2.1.2 Factor-wise least squares means of adjusted Ct values of mRNA expression of genes in different tissues of Aseel chicken**

| Items | LS mean of adjusted Ct values of mRNA expression levels | | |
|---|---|---|---|
| | Tissue | | |
| | Bursa | Spleen | Thymus |
| **Genes** | | | |
| IFN-γ | 14.21 ± 1.19 | 29.34 ± 0.41[a] | 31.00 ± 0.55[a] |
| IL1-β | 11.60 ± 1.39 | 23.80 ± 0.48[c] | 12.10 ± 0.55[c] |
| iNOS | 12.57 ± 1.19 | -2.26 ± 0.41[d] | 24.61 ± 0.47[b] |
| TLR15 | 11.73 ± 1.19 | 26.91 ± 0.41[b] | 11.07 ± 0.47[c] |
| **Sex** | | | |
| Male | 12.47 ± 0.92 | 19.61 ± 0.31 | 19.51 ± 0.39 |
| Female | 12.58 ± 0.84 | 19.28 ± 0.29 | 19.88 ± 0.33 |

*Means within a factor in a column with different superscripts differ significantly (P < 0.05)*

Expression differences among genes were significant (p<0.01) in spleen and thymus tissue of Aseel chicken.

IFN-γ had highest expression followed by TLR15, IL-1β and iNOS in spleen.

In thymus tissue, the IFN-γ had highest expression followed by iNOS, IL1-β and TLR15.

### 4.3.2.2 Kadaknath

Expression differences among (IL1-β, IFN-γ, iNOS and TLR15) genes in different tissues of Kadaknath chicken are presented in Table 4.3.2.2.1 and their least squares means ± SE of adjusted cycle threshold values presented in Table 4.3.2.2.2.

**Table 4.3.2.2.1 Expression differences among IL1-β, IFN- γ, iNOS and TLR15 genes (P values, ANOVA) in different tissues of Kadaknath chicken**

| Source of Variation | df | P Values for specific mRNA gene expression | | |
|---|---|---|---|---|
| | | Tissue | | |
| | | Bursa | Spleen | Thymus |
| Genes | 3 | 0.2818 | <0.0001** | <0.0001** |
| Sex | 1 | 0.2909 | 0.2177 | 0.4572 |

**P<0.01

**Table 4.3.2.2.2 Factor-wise least squares means of adjusted Ct values of mRNA expression of genes in different tissues of Kadaknath chicken**

| Items | LS mean of adjusted Ct values of mRNA expression levels | | |
|---|---|---|---|
| | Tissue | | |
| | Bursa | Spleen | Thymus |
| **Genes** | | | |
| IFN- γ | 13.44 ± 0.84 | 29.48 ± 0.75$^{a}$ | 31.14 ± 0.65$^{a}$ |
| IL1-β | 15.06 ± 0.84 | 25.06 ± 0.75$^{b}$ | 17.45 ± 0.65$^{c}$ |
| iNOS | 15.49 ± 0.84 | 4.93 ± 0 .75$^{c}$ | 26.73 ± 0.65$^{b}$ |
| TLR15 | 13.64 ± 0.84 | 27.69 ±0.75$^{ab}$ | 11.56 ± 0.65$^{d}$ |
| **Sex** | | | |
| Male | 13.94 ± 0.59 | 21.29 ± 0.53 | 21.97±0.46 |
| Female | 14.88 ± 0.59 | 22.28 ± 0.53 | 21.47±0.46 |

*Means within a factor in a column with different superscripts differ significantly (P < 0.05)*

In Kadaknath chicken, the expression differences among genes were significant ($p < 0.01$) in spleen and thymus tissue.

In spleen of Kadaknath, IFN-γ showed highest expression similar to Aseel chicken. However, it was statistically non-significantly more than TLR15, which was again non-significantly more than IL1-β but significantly more than iNOS mRNA.

In thymus tissue, similar pattern was seen as was observed in Aseel chicken i.e. highest expression of IFN-γ, followed by iNOS, IL1-beta and TLR15.

#### 4.3.2.3 WLH chicken

Expression differences among expression of IL1-β, IFN-γ, iNOS and TLR15 genes in different tissues of WLH chicken are presented in Table 4.3.2.3.1 and their least squares means ± SE of adjusted cycle threshold values presented in Table 4.3.2.3.2.

**Table 4.3.2.3.1 Expression differences among IL1-beta, IFN- ã, iNOS and TLR15 genes (P values, ANOVA) in different tissues of WLH chicken**

| Source of Variation | df | P Values for specific mRNA gene expression | | |
|---|---|---|---|---|
| | | Tissue | | |
| | | Bursa | Spleen | Thymus |
| **Genes** | 3 | 0.4869 | <0.0001** | <0.0001** |
| **Sex** | 1 | 0.8039 | 0.1804 | 0.1266 |

**P<0.01

**Table 4.3.2.3.2 Factor-wise least squares means of adjusted Ct values of mRNA expression of genes in different tissues of WLH chicken**

| Items | LS mean of adjusted Ct values of mRNA expression levels | | |
|---|---|---|---|
| | Tissue | | |
| | Bursa | Spleen | Thymus |
| **Genes** | | | |
| IFN-γ | 13.39 ± 0.94 | 29.52 ± 0.87[a] | 31.34 ± 0.74[a] |
| IL1-β | 12.26 ± 0.94 | 23.87 ± 0.87[b] | 13.63 ± 0.74[c] |
| iNOS | 13.59 ± 0.94 | 0.97 ± 0.87[c] | 26.97 ± 0.74[b] |
| TLR15 | 11.77 ± 0.94 | 26.54 ± 0.87[ab] | 9.52 ± 0.74[d] |
| **Sex** | | | |
| Male | 12.87 ± 0.66 | 19.60 ± 0.61 | 20.98 ± 0.52 |
| Female | 12.63 ± 0.66 | 20.85 ± 0.61 | 19.75 ± 0.52 |

*Means within a factor in a column with different superscripts differ significantly (P < 0.05)*

Expression differences among genes were significant ($p \leq 0.01$) in spleen and thymus tissue of WLH chicken.

In the spleen tissue of WLH, the expression patterns of all the four genes followed same trend as was seen in Kadaknath chicken.

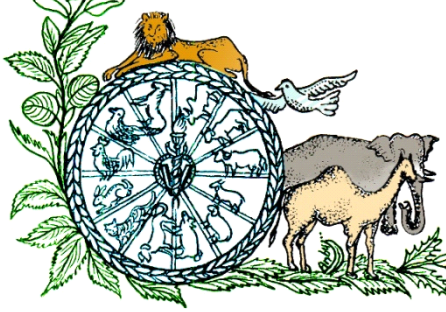
In thymus tissue of WLH, the expression patterns of all the four genes followed same trend as was seen in Aseel and Kadaknath chicken, except that in Aseel chicken the difference between IL1-β and TLR15 expression was statistically non-significant.

In the present study, the level of mRNA expression of all the four genes did not differ significantly in bursa in each of the three genotypes. This is in agreement with the report of Careem *et al.* (2008) who reported that the expression of iNOS mRNA in bursa showed similar pattern as that of IFN-γ.

The moderate level of expression of IFN-γ in all the three genotypes suggested good protection against MDV, although chicks were vaccinated at day old stage as a routine practice at this institute. It has been reported that IFN-γ was up-regulated in the bursa of MDV-infected chickens at early time points post-infection and showed direct inhibitory effect of IFN-γ on MDV *in vitro* (Levy *et al.,* 1999). Furthermore, the chicken strains that are resistant to coccidiosis are reported to have high levels of IFN-γ expression (Byrnes *et al.,* 1993; Lowenthal *et al.,* 1997 and Yun *et al.,* 2000).

The current investigation has expanded knowledge of the basal splenic expression levels of the immune-related genes in different genotypes of chickens. Significant or non-significant differences in the assayed genes suggested little alteration in basal state of expression and the need for expanded studies.

# Summary and Conclusions



# 6 Summary and Conclusions

The immunocompetence status of Aseel, Kadaknath and White Leghorn chicken were evaluated by assessing important parameters related to various facets of immunity such as *in-vivo* antibody response to SRBC, serum lysozyme activity and serum IgG level. The data generated on immunocompetence traits was analyzed by Least-squares analysis of variance

In Aseel, Kadaknath and White Leghorn chicken, the effect of sire and sex was statistically non-significant ($P > 0.05$) on all IC traits. The heritability estimates of IC traits were either very low or could not be estimated in all the three breeds. The genetic correlation ($r_G$) among IC traits could not be estimate. The phenotypic correlations ($r_p$) among IC traits were positive but low in magnitude.

Highly significant differences ($P<0.01$) among breeds for antibody titre against sheep RBCs, Serum Lysozyme level and Serum IgG level were observed. The least square means of Serum Lysozyme level (21.28 ± 0.78 µg/ml) and Serum IgG level (24.23 ± 2.05 µg /µl) were relatively higher in Aseel than Kadaknath (16.91±0.93 and 15.70±2.44, respectively) and WLH chicken (9.42±0.68 and 10.27±1.79, respectively). However, the antibody titre against sheep RBCs was relatively lower in Aseel (10.84 ± 0.18) than the Kadaknath (11.62±0.21) and WLH (11.94±0.15) chicken.

Immune response to Newcastle Disease (ND) vaccine was assased by HI and ELISA tests. The data generated on ND vaccine response was analyzed by Least-squares analysis of using the statistical software

program SPSS16.0 and expressed as mean ± SE. Significant differences among means were determined by Tukey's HSD test at $P < 0.05$.

HI titres showed significant ($P<0.01$) differences among dpi's in Aseel and Kadaknath chicken while in WLH chicken differences among dpi's were non-significant ($P>0.05$). In Aseel native chicken, 7 dpi exhibited some maternal response. Subsequently, from 14 dpi onwards, the trend was increasing, highest being at 42 dpi. In Kadaknath native chicken, first peak was observed at 14 dpi, the titre decreased at 21 dpi and showed continuously increasing trend thereafter. In WLH chicken, higher response was observed at 7 dpi and 21 dpi and thereafter decreasing trend was seen.

ELISA titres showed significant ($P\leq0.01$) differences among dpi's in Aseel and Kadaknath chicken as well as in WLH chicken ($P<0.05$). In Aseel, There was declining trend till 14 dpi and thereafter an increasing trend in antibody response was observed. The highest tire was found at 42 dpi. In Kadaknath chicken, there was an increasing trend in the vaccine response from 7 dpi to 42 dpi. The highest tire was found at 42 dpi. In WLH chicken, there was declining trend until 28 dpi and inclining trend thereafter. The highest antibody titre was found at 7 dpi that revealed the vaccination of day-old chicks is very important to enhance the maternal derived antibody response.

Comparison of HI titres and ELISA titres revealed similar pattern in Aseel chicken while in Kadaknath and White Leghorn chicken trend was not as consistent as in Aseel.

Genotype differences in vaccine response to NDV vaccine using HI test revealed significant ($P\leq0.05$) differences at 7 dpi, 35 dpi and 42 dpi only. At 7 dpi WLH chicken showed highest antibody titre (8.48±0.28) followed by Aseel and Kadaknath chicken (8.10±0.22 and 7.32±0.16, respectively) while at 35 and 42 dpi Kadaknath chicken (8.43±0.17 and 9.14±0.16, respectively) showed highest titre followed by Aseel (8.40±0.16 and 8.94±0.19, respectively) and WLH chicken (7.68±0.29 and 7.73±0.29, respectively).

Genotype differences in vaccine response to NDV vaccine using ELISA test revealed significant differences ($P \leq 0.05$) at 7 dpi, 14 dpi and 42 dpi only. At 7 dpi, WLH chicken showed highest antibody titre (3.20±0.07) followed by Aseel (2.20±0.11) and Kadaknath chicken (2.08±0.16) while at 14 dpi WLH chicken showed highest antibody titre (2.93±0.09) followed by Kadaknath (2.16±0.14) and Aseel (2.12±0.16) chicken. At 42 dpi Aseel (3.04 ± 0.08) showed highest titre followed by WLH (2.79±0.09) and Aseel chicken (2.66±0.12).

The relative expression of four immunity related genes (IL1-β, IFN-γ, iNOS and TLR15) were assessed by quantitative reverse transcription PCR (qRT-PCR). β-Actin gene used as reference gene. The data analysis of gene mRNA expression was performed with an ANOVA model using JMP software (ver 9.1) of SAS Institute, 2010.

In bursa tissue, neither genotype nor sex had significant effect ($p > 0.05$) on the expression of four genes studied. Genotype showed little differences ($p < 0.11$) in expression of IL1-beta.

In spleen tissue, genotypic differences among Aseel, Kadaknath and white Leghorn chicken were significant ($p \leq 0.05$) on iNOS mRNA expression and to some extent on TLR15 ($P=0.096$). The mRNA expression of iNOS gene in spleen tissue was highest in Kadaknath chicken followed by WLH and Aseel chicken. Sex had no significant effect on mRNA expression of any of the genes studied.

In thymus tissue, genotypic differences among Aseel, Kadaknath and white Leghorn chicken were significant ($p \leq 0.01$) on mRNA expression of IL1-β gene only. Sex effect was non-significant on mRNA expression of three genes (IL1-β, IFN-γ, and iNOS) but significant ($p \leq 0.05$) on TLR15. Kadaknath chicken showed highest mRNA expression of IL1-β gene followed by WLH and Aseel chicken. TLR15 mRNA expression was higher in male than female.

The mRNA expression of all the four genes varied significantly ($p \leq 0.01$) among tissues in Aseel, Kadaknath and white Leghorn chicken. The mRNA expression of IL1-β and TLR15 were highest in spleen

whereas IFN-γ and iNOS were highest in thymus tissue of all the breeds studied.

In all the three genotypes, the significant expression differences among genes were observed ($p < 0.01$) in spleen and thymus tissues. In all three genotypes, IFN-γ gene more expreesed in spleen and thymus tissue than other immunity related genes taken in the study.

Higher status of immunocompetence and vaccine response to NDV in native chicken may be exploited by their selective introgression in high yielding chicken germplasm. Owing to the variable basal expression patterns of immunity relate genes; present study opens the doors for studies on genetics of host resistance to pathogens.

Following conclusions were drawn

- Assessment of Response to NDV vaccine through Haemagglutination (HI) test revealed that differences among dpi's in Aseel and Kadaknath chicken were significant ($P<0.01$) while in WLH chicken, the differences among dpi's were non-significant ($P>0.05$). Genotype differences were significant ($P<0.05$) at 7 dpi, 35 dpi and 42 dpi only. At 7 dpi WLH chicken showed highest antibody titre, non-significantly different from Aseel but significantly different from Kadaknath chicken. While at 35 dpi, the titre in Kadaknath revealed highest titre, non-significantly different from Aseel but significantly different from WLH chicken. The difference between Aseel and WLH was not significant. At 42 dpi, Kadaknath chicken demonstrated highest titre, non-significantly different from Aseel but significantly different from WLH chicken.
- Assessment of Response to NDV vaccine through ELISA test showed significant ($P<0.01$) differences among dpi's in Aseel, Kadaknath and WLH chicken ($P<0.05$). Genotype differences were significant ($P<0.05$) at 7 dpi, 14 dpi and 42 dpi only. At 7 and 14 dpi, WLH chicken showed highest antibody titre followed by Aseel and Kadaknath chicken; Aseel and Kadaknath did not differ significantly. At 42 dpi, Aseel showed highest titre, non-significantly different from WLH but significantly different from

Kadaknath; the difference between Kadaknath and WLH were non-significant.

- Expression analysis of four immunity related genes (IL-1beta, IFN-γ, iNOS and TLR-15) in bursa tissue revealed that neither genotype nor sex had significant effect ($p > 0.05$) on gene expression. Genotypes showed little differences ($p<0.11$) in expression of IL1-beta.
- Expression analysis in spleen tissue revealed that genotypic differences among Aseel, Kadaknath and white Leghorn chicken were significant ($p\leq0.05$) on iNOS mRNA expression and to some extent on TLR15 ($P=0.096$). The mRNA expression of iNOS gene in spleen tissue was highest in Kadaknath chicken followed by WLH and Aseel chicken. Sex had no significant effect on mRNA expression of any of the genes studied.
- Expression analysis in thymus tissue resolved that the genotypic differences among Aseel, Kadaknath and white Leghorn chicken were significant ($p\leq0.01$) on mRNA expression of IL1-β gene only. Kadaknath chicken showed highest mRNA expression of IL1-β gene followed by WLH and Aseel chicken. TLR15 mRNA expression was higher in male than female ($p< 0.05$). Sex effect was non-significant on mRNA expression of other three genes (IL1-β, IFN-γ, and iNOS).
- The mRNA expression of all the four genes (IL-1beta, IFN- γ, iNOS and TLR-15) varied significantly (p d" 0.01) among tissues in Aseel, Kadaknath and white Leghorn chicken. The mRNA expression of IL1-β and TLR15 were highest in spleen whereas IFN-γ and iNOS were highest in thymus tissue of the three breeds.
- In all the three genotypes, the significant expression differences among genes were observed ($p < 0.01$) in spleen and thymus tissues only, wherein IFN-γ gene was more expressed in spleen and thymus tissue as compared to other three immunity related genes.

# Mini Abstract

# 7 Mini Abstract

In the present study, the immunocompetence status of Aseel, Kadaknath and White Leghorn chicken, and their immune response to Newcastle Disease vaccine (NDV) were assessed. The relative expression of four immunity related genes (IL-1β, IFN-γ, iNOS and TLR15) were assessed by quantitative reverse transcription PCR (qRT-PCR). The results concluded that the influence of sire and sex on IC traits were non-significant ($P<0.05$) in all the breeds studied, however, the genotypes differed significantly ($P<0.01$) for IC traits. Lysozyme level and Serum IgG level were relatively higher in Aseel than the other two genotypes. HI titres against NDV vaccine at different dpi differed significantly ($P<0.01$) in Aseel and Kadaknath chicken. Highest HI titre was observed at 42 dpi in Aseel and Kadaknath chicken while 7 dpi in WLH chicken. ELISA titres against NDV vaccine differed significantly ($P<0.01$) in Aseel, Kadaknath and WLH ($P<0.05$) chicken at different dpi. Highest ELISA titre was observed at 42 dpi in Aseel and Kadaknath chicken while 7 dpi in WLH chicken that was similar to HI titres. In spleen tissue, iNOS transcripts was more in Aseel than WLH and Kadaknath chicken, while in thymus tissue, the mRNA expression of IL1-β gene was more in Aseel than the WLH and Kadaknath chicken. TLR15 mRNA expression was higher in female than male. The mRNA expression of all the four genes varied significantly ($p<0.01$) among tissues in Aseel, Kadaknath and white Leghorn chicken. The transcripts levels of IL1-β and TLR15 were highest in spleen, whereas IFN-γ and iNOS highest in thymus tissue of all the breeds studied. In all the three genotypes, the expression differences among genes were observed significantly ($p<0.01$) in spleen and thymus tissues, while, IFN-γ gene was more expressed in spleen and thymus tissue than other immunity related genes taken in the study. From the above study it can be concluded that, higher status of immunocompetence and vaccine response to NDV in native chicken may be exploited for their selective introgression in high yielding chicken germplasm. Owing to the variable basal expression patterns of immunity relate genes; present study opens the doors for study on genetics of host resistance to pathogens.

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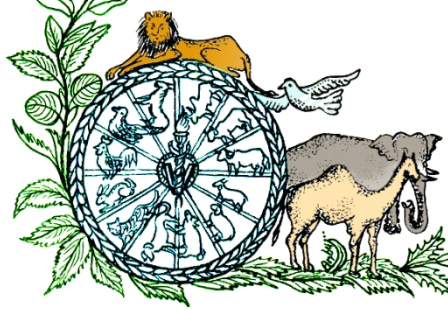

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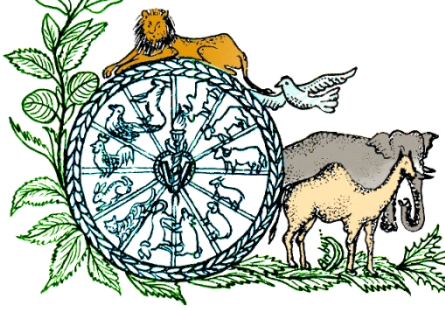

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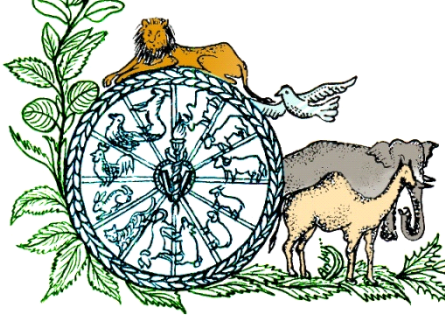
# Appendix



# Appendix

**Reconstitution of dNTP's (pH=7.0)**

Working solution of dNTP mix with 10mM of each dNTP from 100 mM of each dNTP stock.

10 µl of each dNTP is taken and the volume is made upto 100 µl *i.e.,* 10 µl of dATP + 10µl of dGTP + 10µl dTTP +10µl dCTP + 60µl of td$H_2O$ (nuclease free). The effective concentration of each dNTP becomes 10 mM in the mix.

$$N_1V_1 = N_2V_2$$

$$10\ mM \times V_1 = 200\mu M \times 50\mu l$$

(Final concentration should be 200µM of each dNTP in 50µl)

$$x = 1\mu l$$

Therefore in a 50 µl reaction to have 200 µM of each dNTP 1µl of working solution is added. For getting a concentration of 300 µM in 50µl reaction 1.5µl of working solution is added.

**Working solution of 2.5 mM of each dNTP from 10 mM of each dNTP stock**

10 µl of dATP + 10 µl dGTP + 10 µl of dTTP + 10 µl of dCTP are added in an eppendorf making the effective concentration of each dNTP to 2.5 mM.

$$N_1V_1 = N_2V_2$$

$$2.5mM \times V1 = 200\mu M \times 50\mu l$$

(Final concentration should be 200µM of each dNTP in 50µl)

$$V_1 = 4\mu l$$

Therefore in a 50 µl reaction to have 200 µM of each dNTP 4µl of working solution is added.

**Primer Reconstitution**

From the stock of 1000 pM/µl, working solution of 20 pM/µl is prepared by taking 1µl of stock in an eppendorf tube and making up

the volume to 50µl. In a PCR reaction 1µl of working solution is added for a 50µl reaction to get an effective concentration of 0.4 µM.

$$N_1V_1 = N_2V_2$$

20 pM X V1 = 0.4 µM X 50µl

$V_1 =$ (0.4 µM X 50µl) /20 pM

= 1µl

Similarly if 1.5µl is added the effective concentration becomes 0.6µM in 50µl reaction.

$$N_1V_1 = N_2V_2$$

20 pM X V1 = 0.6 µM X 50µl

$V_1 =$ (0.6 µM X 50µl)/ 20 pM

= 1.5 µl

**$MgCl_2$**

1µl of 25 mM is added for a 50 µl reaction to get an effective concentration of 0.5 mM.

$$N_1V_1 = N_2V_2$$

25 mM X V1 = 0.5 mM X 50µl

$V_1$ = 1µl

**10 X buffer with $MgCl_2$ or without $MgCl_2$**

The buffer is diluted to make 1X in the PCR reaction. The buffer may contain $MgCl_2$. The requirement of the $MgCl_2$ can be taken care by the buffer. If the PCR reaction needed more concentration than in the buffer it should be provided additionally.

**10X Taq buffer**

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

Store at -20ºC.

Use at a final concentration of 2.5 mM.

**Taq DNA Polymerase enzyme** (3 units/µl)

Store at -20ºC and use at a final concentration of 0.75 U.

**TBE (Tris, boric acid, EDTA) buffer (5X)**

| | |
|---|---|
| Tris base | 54.0 g |
| Boric acid | 27.5 g |
| 0.5 M EDTA | 20.0 ml |
| Adjust pH to 8.0 | |

**6 X loading dye for Agarose gel**

| | **Type-1** | **Type-2** |
|---|---|---|
| Bromophenol blue | 0.25% | 0.25% |
| Xylene Cyanol | 0.25% | - |
| Sucrose in DW | 40% | 40% |
| Mix and store at 4°C | | |

These gel loading buffers serve three purposes: They increase the density of the sample ensuring that the DNA drops evenly into the well; they add colour to the sample, thereby simplifying the loading process; and they contain dyes that, in an electric field, move toward the anode at predictable rates. Bromophenol Blue migrates through agarose gels approximately 2.2-fold faster than Xylene Cyanol FF, independent of the agarose concentration. Bromophenol Blue migrates through agarose gels run in 0.5 X TBE at approximately the same rate as linear double-stranded DNA 300 bp in length, whereas Xylene Cyanol FF migrates at approximately the same rate as linear double stranded DNA 4 kb in length. These relationships are not significantly affected by the concentration of agarose in the gel over the range of 0.5% to 1.4%.

**20,000 X Ethidium Bromide**

| | |
|---|---|
| Ethidium Bromide | 10 mg |
| Autoclaved $dH_2O$ | 1000 ml |

Wrap in an aluminium foil and store at room temperature in a dark place (as Et.br. is photosensitive).

Function of Et.br.- acts as intercalating agent to make complex with DNA/RNA molecules which illuminates when exposed over UV light.

**100bp DNA ladder (0.1µg/µl)**

Store at 4ºC for within-use or at -20ºC for ever.

**Dibasic buffer (0.066M, pH 6.3)**

Contains

| | |
|---|---|
| (i) $Na_2HPO_4$ (1M) | 100 ml |
| (ii) $Na_2H_2PO_4$ (1M) | 100 ml |
| (iii) $dH_2O$ | 800 ml |

Dissolve 14.196 g $Na_2HPO_4$ in 100 ml of $dH_2O$ and 15.601 g $Na_2H_2PO_4$ in 100 ml of $dH_2O$ for preparing dibasic buffer and store at 4ºC.

**0.1 M Tris HCl (pH 8.0)**

Tris HCl -157.6 g
Distilled water upto 1000 ml.
Adjust pH to 8.0 with NaOH pellets.
Autoclave in 100 ml batches.

**Standard Lysozyme (2µg/µl) stock solution**

Dissolve 2.0 mg of standard lysozyme in 1000 µl of dibasic buffer.

***Micrococcus lysodieketicus* (1mg/ml) bacterial stock solution**

Mix 50 µl of *M. lysodieketicus* in 1000 µl of $dH_2O$.

**Rabbit Anti-chicken IgY**

Use as such @ 35 µl/ml of 0.1M Tris HCl required for gel preparation in lysoplate.

**Standard Chicken IgG (25 mg/ml) stock solution**

Dilute 10 mg of standard chicken IgG in to 400 µl of 0.1M Tris-HCl.

**0.2% Coomassie Brilliant Blue (CBB)**

Dissolve 200 mg of CBB in 2 ml of methanol and add 2 ml of Acetic Acid followed by making the final volume upto 100 ml with distilled water.

**Destaing solution (1000 ml)**

| | |
|---|---|
| Methanol | 3 parts |
| Acetic Acid | 1 part |

Distilled water- 6 parts and mix well.

# Curriculum Vitae

**Dr. Kokate Laxmikant Sambhaji**

## EDUCATIONAL QUALIOFICATION

| Qualification | Board/ University | Year of Completion | % of marks |
|---|---|---|---|
| M. V. Sc (AGB) | National Dairy Research Institute, Karnal, Haryana | 2009 | 76 |
| B.V. Sc & A. H | MAFSU, Nagpur, Maharashtra | 2007 | 65 |
| H.S.C | Divisional Board, Latur, Maharashtra | 2002 | 56 |
| S.S.C | Divisional Board, Latur, Maharashtra | 1999 | 71 |

## PERSONAL INFORMATION

| | |
|---|---|
| **Date of Birth** | 3 rd March, 1984 |
| **Sex** | Male |
| **Marital status** | Unmarried |
| **Nationality** | Indian |
| **Language known** | Marathi, English & Hindi |
| **Phone** | +91-8954534476 |
| **E-mail** | kokatels@gmail.com |
| **Address** | Sumedh Nagar, Purna Road, Taroda, Nanded, Tq. & Dist. Nanded, Maharashtra, India. |
| **Father's Name** | Mr. Kokate Sambhaji |
| **Mother's Name** | Mrs. Kokate Lalita |