FINAL PROGRESS REPORT

(Terminal Report: 2013-2016)

RESEARCH PROJECT

ON

"RELATIONSHIP OF MHC GENES POLYMORPHISM WITH COCCIDIAL RESISTANCE IN CHICKEN"

PRINCIPAL INVESTIGATOR

Dr. M. S. Thakur
Assistant Professor/Scientist

DEPARTMENT OF ANIMAL GENETICS AND BREEDING
COLLEGE OF VETERINARY SCIENCE & ANIMAL HUSBANDRY,
NANAJI DESHMUKH VETERINARY SCIENCE UNIVERSITY,
JABALPUR (M.P.) 482001

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FINAL PROGRESS REPORT

(From 01/04/2013 to 31/08/2015)

1. **Title of the project** : **Relationship of MHC genes polymorphism with coccidial resistance in chicken**.

2. Full name , designation and nature of appointment of the **Principal Investigator** (Tel.no.& e-mail) :

Dr. Mohan Singh Thakur
Assistant Professor/Scientist
Deptt. of Animal Genetics & Breeding,
College of Vety. Sci. & A.H., Jabalpur M.P.).
Mob. No. 08989126042
Email : drmohansingh@gmail.com

3. Full name and designation of the **Co - Investigator /s** (Tel.no.& e-mail)

Dr. Giridharilal Das
Associate Professor,
Department of Veterinary Parasitology,
College of Vety. Sci. & AH., Jabalpur.
Mob. No. 09425963518
Email address: gdas7@yahoo.com

4. Postal Address of the Principal investigator and Co- investigator/s :

Department of Animal Breeding & Genetics,
College of Veterinary Science & A. H.,
N.D.V.S.U., Jabalpur (M.P.)-482001

5. Name of the institution(s)/Organization in which the project is carried out : **College of Veterinary Science & Animal Husbandry Nanaji Deshmukh Veterinary Science University, Jabalpur (M.P.)-482001**

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9. Total Sanctioned Budget : **Rs. 12.33 Lakhs**

INTRODUCTION:

Parasitic infection of the intestinal tract of domestic fowl is a major veterinary health problem worldwide. Among the parasitic infections coccidiosis has the most devastating bearing on poultry industry. Avian coccidiosis is an intestinal disease caused by apicomplexan protozoa belonging to at least seven different species of *Eimeria.* The disease has a great economic impact in poultry productions partly due to resistance of the organisms to anticoccidial drugs. Cross and multiple resistance of anticocidial drugs occurred when these drugs were tested on various *Eimeria* organisms.

The most common and pathogenic species that affects the poultry industry is *E. tenella*, resulting in 100% morbidity and a high mortality due to extensive damage of the digestive tract. The disease is manifested clinically by intestinal haemorrhage, malabsorption, diarrhoea, reduction of body weight gain due to inefficient feed utilization, impaired growth rate in broilers and reduced egg production in layers.

In India, coccidiosis is a serious problem and is one of the biggest causes of economic losses in poultry. It inflicts heavy mortality every year mainly in broilers and also in growers raised on deep litter. Approximately 80% of the entire cost is due to the direct effects on mortality, weight gain and feed conversion, and 20% is due to the cost of chemoprophylaxis (Bera *et al*., 2010). Improving disease resistance in poultry by direct selection or by selecting for immune response is hardly feasible due to quantitative nature of these traits, their low heritability and the difficulties associated with reliable measurements.

The broiler chicks in our country are usually reared under intensive conditions that are conducive to any infections by opportunistic pathogens. Among the infectious diseases of poultry, the coccidiosis caused by a protozoan parasite (*Eimeria species*), which is one of the most detrimental and lethal disease. It renders the poultry farmers to experience major economic losses. The diseases results in tissue damages, reduced feed conversion and body weight, bleeding, high morbidity and mortality of the chicks. The mortality of birds is usually attributed to either severe caecal haemorrhage and excessive blood loss alone or coupled with toxic factors and bacterial products. However, the continuous and indiscriminate use of chemicals worldwide may limit the usefulness of anticoddidial drugs as there is always an apprehension of emergence of drug resistant strains of *Eimeria* species and there are chances of frequent failure of medication against

the disease. Hence, the disease continues to be the serious and economically important health problem of broiler chicks for poultry farmers

The Major Histocompatibility Complex (MHC) in chickens, initially known as 'B Complex' (Briles *et al.*, 1950) is an extensively polymorphic erythrocyte antigen or blood group system and is perhaps the best characterized family of host genes that modulates response to a variety of antigens and pathogenic challenges. For genetic improvement of economic traits and diseases resistance, breeder can identify or use identified haplotypes of MHC conferring resistance to particular diseases for optimum gains. The chicken MHC is commonly identified with polyclonal antisera produced by immunizations between birds having different haplotypes (Briles and Briles, 1982 and Juul-Madesen *et al*., 1993). The MHC haplotype nomenclature was standardized initially using serologic reagents (Briles *et al*., 1982). But traditional serology for MHC identification has several limitations, including subjectivity in interpretation of serological reactions and expertise required for production of new reagents (Wang *et al.*, 2014). In addition, the cost and time involved in serological reagent development are huge. However, allelic typing can be simplified using polymerase chain reaction using sequence specific primers (PCR-SSP). With this method large number of birds can be easily analyzed for allelic variation without the use of labeled probes for MHC Class II molecules. Therefore, information of MHC can be used as a means of altering allelic frequencies selectively to improve correlated traits, primarily disease resistance and immune response (Kaufman and Lamont, 1996).

But there is a dearth of literature available on the MHC typing and its association with coccidial resistance in synthetic breed/lines. Therefore, the present study was proposed with the following objectives.

1. To study the polymorphism at MHC genes locus in five identified commercial lines and Kadaknath breeds of chicken.

2. To study genetic differences in susceptibility due to coccidial challenge in five identified commercial lines and Kadaknath breeds of chicken.

3. To determine the association of MHC genes with growth traits in five identified commercial lines and Kadaknath breeds of chicken.

SUMMARY OF THE PROJECT:

As per the mandate and approved objectives of project, the research work has been completed in the five identified commercial breed/lines (**Cobb**, **Hubbard**, **Caribro-91**, **Naked Neck** & **Jabalpur Dual Coloured Birds**) and **Kadaknath** breed of chicken (Plates 1 & 2). The salient findings of progress report of research project have been discussed bellow as per the approved objectives:

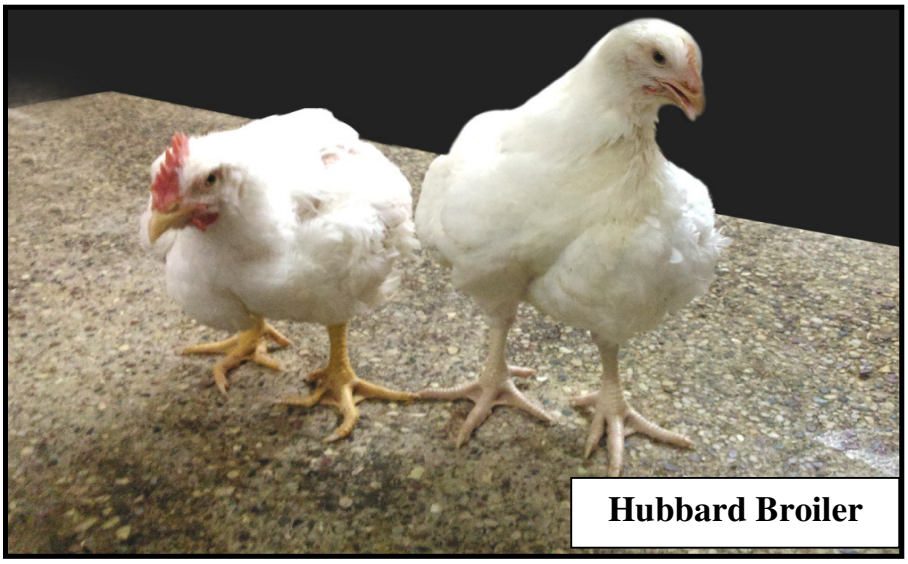
1. Experimental Birds of different breed/lines

The present investigation was conducted on **Kadaknath**, **Caribro-91**(Cari Vishal), **Jabalpur Dual Coloured**, **Hubbard**, **Naked Neck** birds and **Cobb** broiler chicken under the research project funded by Madhya Pradesh Biotechnology Council, Bhopal, Department of Animal Genetics and Breeding, College of Veterinary Science and Animal Husbandry, Nanaji Deshmukh Veterinary Science University (NDVSU), Jabalpur. Sixty day old broiler chicks each of Kadaknath, Jabalpur Dual Coloured, Hubbard and Cobb broiler chicks procured from the hatchery were used in the study while fertile eggs of Caribro-91(Cari Vishal) and Naked Neck broiler chicken were brought from Central Avian Research Institute, Izzatnagar (U.P.) and were hatched in the Department of Poultry Science, College of Veterinary Science and Animal Husbandry, NDVSU, Jabalpur. The details of the experimental birds are given in Table 01.

Table 01. Experimental materials

S. No.	Breed	Number of birds	Source
1.	Kadaknath	60	Department of Poultry Science, College of Veterinary Science, Jabalpur, M.P.
2.	Caribro-91(Cari Vishal)	60	Central Avian Research Institute, Izzatnagar, U. P.
3.	Cobb broiler	60	Phoenix Group, 1333/1, Narmada Road, Jabalpur
4.	Naked Neck	60	Central Avian Research Institute, Izzatnagar, U. P.
5.	Jabalpur Dual Coloured	60	Department of Poultry Science, College of Veterinary Science, Jabalpur, M.P.
6.	Hubbard	60	Skylark Hatcheries, Naini, Allahabad, U.P.

Plate 1: Different breeds/lines of chickens



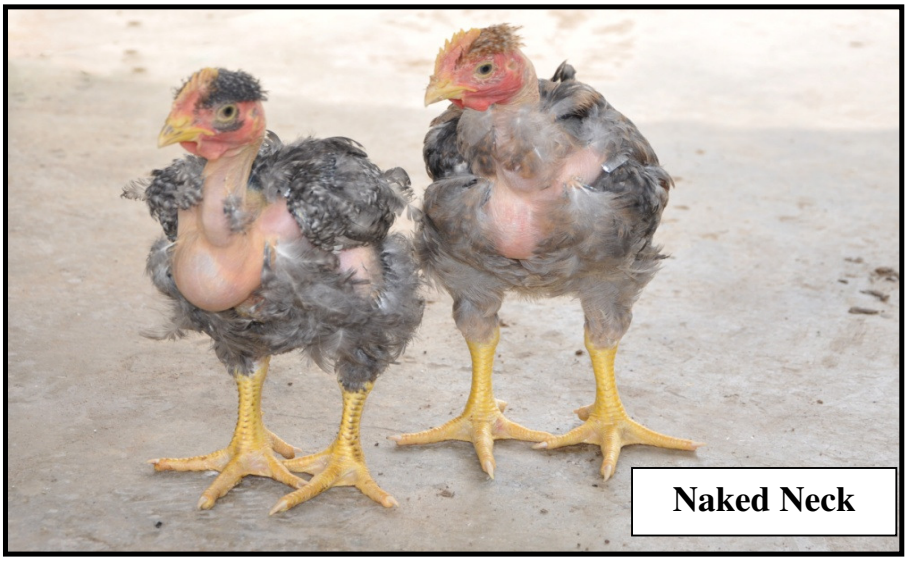


Plate 2: Different breeds/lines of chickens

2. Housing and Management of Experimental birds

Fumigation of brooder house: The battery brooder house was fumigated by using a combination of 35 ml of formalin (40 per cent formaldehyde) and 10 g potassium permanganate solution for one cubic meter of space. The brooder batteries were disinfected with 2.5 per cent phenol solution and by the using of a blowlamp.

Housing and management: On arrival (day 1) they were wing-banded, weighed and randomly allocated to the treatments. During the entire experimental periods there was continuous lighting. The basal diet used did not contain growth promoters or coccidiostats. The birds were maintained in the brooder batteries kept inside a well ventilated room in the college experimental poultry shed unit under proper coccidian free conditions. The usual precautions for raising chicks under coccidian free conditions were observed. The faecal samples of these birds were examined for the presence of *Eimeria* spp. oocysts, if any. It was ascertained before the start of the experiment that birds were free from any coccidial infection. The chicks after brooding were transferred to deep litter unit.

3. Feeding and watering of Experimental birds

The standard chick diet free of any anti-coccidial additives and sanitized tap water were given to the birds *ad lib* throughout the experiment. The feed was provided on the paper and water in aluminium pan during the first four days of brooding and thereafter, feed and water were served to them in linear chick feeders and waterers fitted outside the brooder batteries and then in plastic feeders and waterers in deep litter unit.

4. Collection and Preparation of *Eimeria tenella* dose for challenge study

4.1. Field isolates of *Eimeria tenella*

Field isolates of *Eimeria spp.* were collected from Central Poultry Diagnostic Laboratory (Phoenix Group) 1333/1, Narmada Road, Jabalpur and unorganized farms in and around Jabalpur. The oocysts of *Eimeria tenella* were collected from the caeca of infected broiler chicks. The *Eimeria tenella* infection was identified by the appearance of predominant caecal lesions and presence of characteristic schizonts and gametocytes in the freshly prepared smears of caecal mucosa (Davies *et al.,* 1963). After proper identification, the oocysts collected from the caeca of these birds were processed for inoculums of *Eimeria tenella* in the laboratory.

4.2. Preparation of *Eimeria tenella* inoculum

The *Eimeria tenella* inoculums were prepared separately in three different stages i.e. harvesting of oocysts, sporulation of oocysts and storage of inoculums.

Harvesting of oocysts: The infected caeca collected were kept in 2.5 per cent potassium dichromate solution @ 20 ml/g of tissue separately and oocysts were harvested as per the method described by Davies *et al.* (1963) with certain modifications as follows:

The caecal contents of infected birds were homogenized in 2.5 per cent potassium dichromate solution and the homogenate was filtered through muslin cloth to remove large debris. The filtrate was then sieved sequentially through three sieves of different mesh sizes i.e. 40, 80 and 100 meshes per linear inch, respectively. The sieved material was then centrifuged in 50 ml plastic centrifuge tubes at 1500 rpm for 2 minutes. The pellet of centrifugate was subjected to floatation technique in saturated sodium chloride solution to obtain the oocysts. The surface layer in each tube was then pipetted out into a large volume of tap water to dilute the salt solution and allowed the oocysts to sediment overnight. The supernatant was discarded and the sediment was centrifuged again to remove water.

Sporulation of oocysts: The sediment of oocysts was resuspended in 2.5 per cent solution of potassium dichromate for sporulation. The sporulation was carried out by distributing the oocysts' suspension in 2.5 per cent potassium dichromate solution in shallow layers (3-5 mm) in large petri plates (6 inch in diameter) which were then placed in a BOD incubator at 29 + 1^0C. The forced aeration of the suspension was carried twice daily in order to prevent the oocysts under sporulation was from drying. The 2.5 per cent potassium dichromate solution was added repeatedly till the sporulation of oocysts was completed. The oocysts were regularly examined daily for their sporulation upto 52 hrs.

Storage of inoculums: The sporulated oocysts were transferred into conical flasks (250 ml) having sufficient potassium dichromate solution (2.5%). These flasks were then labeled and stored at 4^0C till further use. The number of sporulated oocysts in the suspension was estimated and the volume was adjusted to contain the 10,000 sporulated oocysts/ml of suspension using McMaster counting chamber.

5. PARAMETERS FOR DISEASE RESISTANCE TRAITS

5.1 Body weight gain

The chicks from all the experiment groups were weighed at day old and at weekly interval upto 8 weeks of age. The average weekly body weights in different genetic groups under investigation are given in Tables 02 to 07.

Table 02. Weekly body weights of Kadaknath breed

Age (weeks)	Control	T_1 (10x 10^3)
1 week	54.68	53.71
2 weeks	70.03	70.01
3 weeks	109.40	107.26
4 weeks	176.00	154.94
5 weeks	255.56	213.87
6 weeks	344.28	282.64
7 weeks	472.28	375.41
8 weeks	658.71	518.21

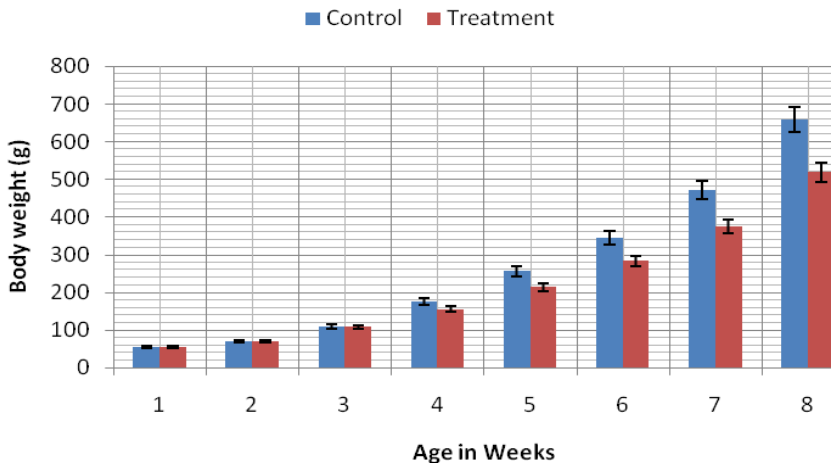


Figure 1: Average weekly body weight (g) in Kadaknath breed

The difference in the body weight of Kadaknath birds under control and treatment group upto 3rd week of age was non-significant. As shown in Figure 1, the lower mean body weight was observed in treatment group as compared to control group from 4 to 8 week of age.

Table 03. Weekly body weight of Jabalpur Dual Coloured birds

Age (weeks)	Control	T_1 (10x 10^3)
1 week	67.61	70.58
2 weeks	85.68	87.13
3 weeks	139.50	141.75
4 weeks	211.18	196.66
5 weeks	305.12	276.75
6 weeks	412.14	369.17
7 weeks	576.67	473.92
8 weeks	777.58	705.82

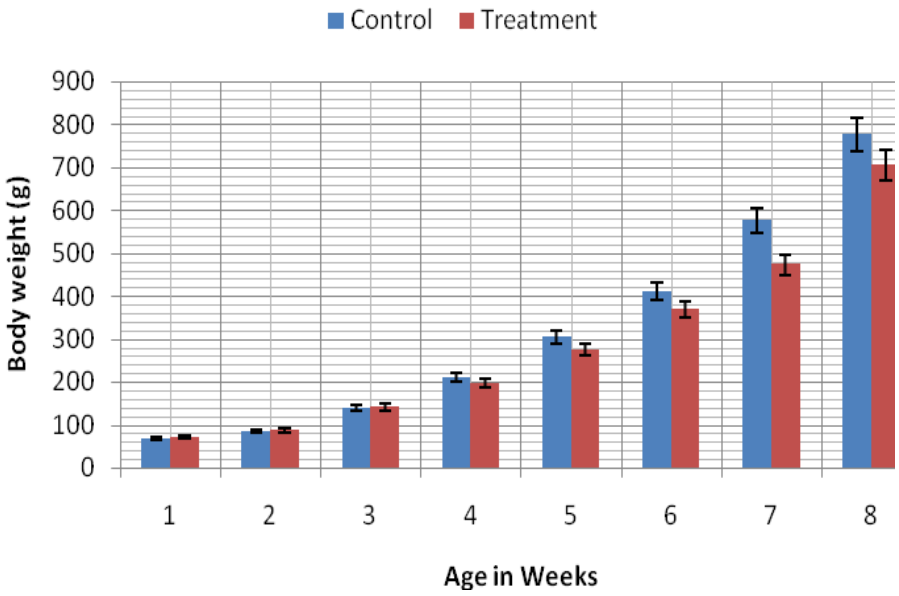


Figure 2: Average weekly body weight in Jabalpur Dual Colored birds

Similarly in Jabalpur colored birds, the difference in the body weight of birds under control and treatment group upto 3rd week of age was non-significant. However, after infection of *Eimeria tenella* with dose of 10,000 sporulated oocycts, the reduction in body weight was observed in treatment groups (Figure 2) from 4 to 8 weeks of age. The average body weight in control and treatment groups of Jabalpur dual colored birds were ranged from 67.61 to 777.58 g and 70.58 to 705.82 g, respectively.

Table 04. Weekly body weight of Cobb broilers

Age (weeks)	Control	T_1 (10x 10^3)
1 week	134.29	141.39
2 weeks	337.10	365.17
3 weeks	687.06	736.56
4 weeks	1233.00	1123.56
5 weeks	1518.53	1460.39
6 weeks	1945.35	1596.72
7 weeks	2064.82	1790.82
8 weeks	2161.43	1914.73

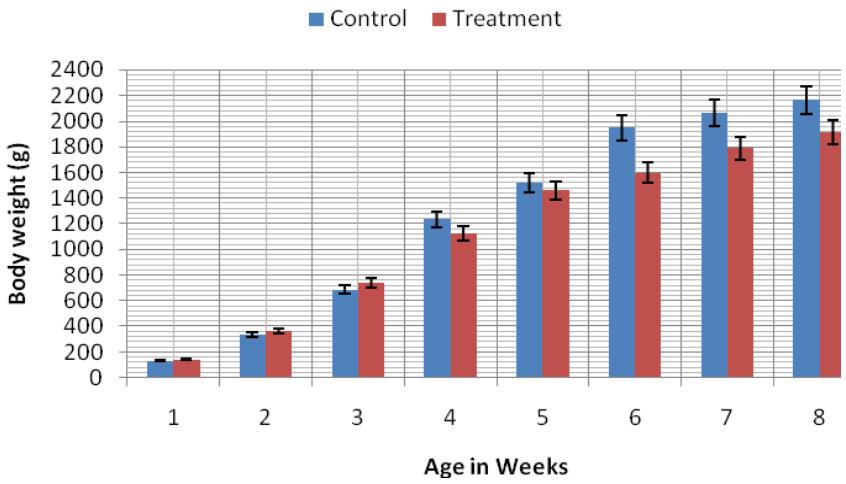


Figure 3: Average weekly body weight (g) in Cobb broiler

The average body weight in control and treatment groups of Cobb broiler birds were ranged from 134.29 to 2161.43 g and 141.39 to 1914.73 g, respectively. As shown in Figure 3, the higher mean body weight was observed in control group as compared to treatment group from 4 to 8 week of age.

Table 05. Weekly body weight of Hubbard broilers

Age (weeks)	Control	T_1 (10x 10^3)
1 week	65.41	63.11
2 weeks	205.24	294.11
3 weeks	432.41	488.33
4 weeks	695.24	682.78
5 weeks	1037.47	1015.28
6 weeks	1541.85	1247.28
7 weeks	1813.78	1456.50
8 weeks	2045.42	1696.57

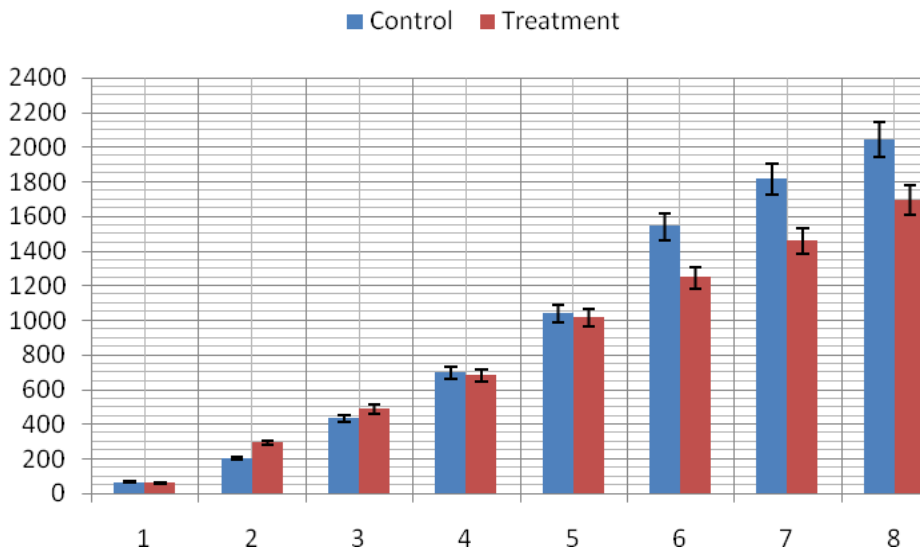


Figure 4: Average weekly body weight (g) in Hubbard broiler

In Hubbard broiler birds the average body weight in control and treatment groups of were ranged from 65.41 to 2045.42 g and 63.11 to 1696.57 g, respectively. As shown in Figure 4, the higher mean body weight was observed in control group as compared to treatment group from 4 to 8 week of age.

Table 06. Weekly body weight of Naked Neck breed

Age (weeks)	Control	T_1 (10x 10^3)
1 week	113.80	115.24
2 weeks	230.70	233.85
3 weeks	367.73	365.69
4 weeks	654.12	593.33
5 weeks	973.73	846.91
6 weeks	1136.10	1023.77
7 weeks	1382.90	1237.56
8 weeks	1624.85	1426.11

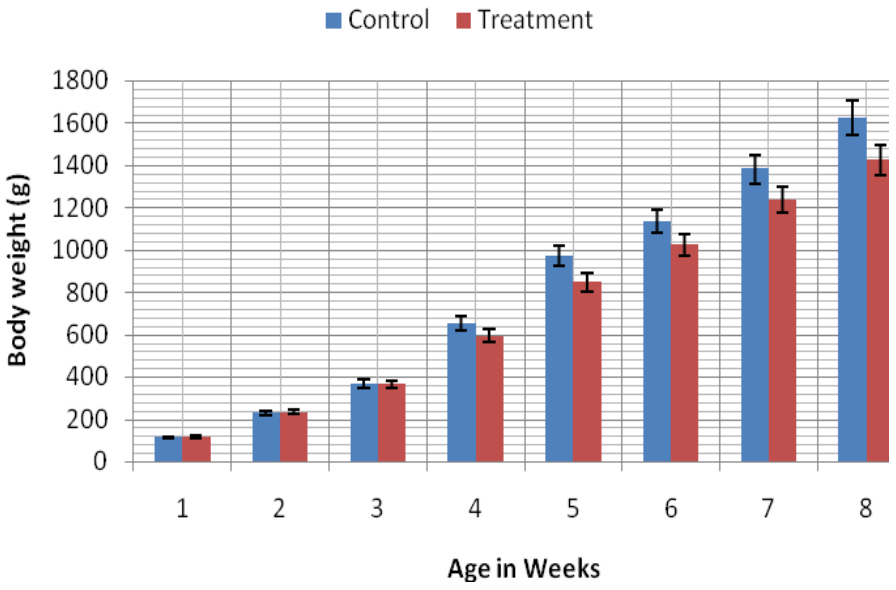


Figure 5: Average weekly body weight (g) in Naked Neck breed

In Naked Neck breed the average body weight in control and treatment groups were ranged from 113.80 to 1624.85 g and 115.24 to 1426.11 g, respectively. As shown in Figure 5 the birds under controlled showed superior growth as compared to treatment group from 4 to 8 week of age.

Table 07. Weekly body weight of Caribro-91 (Cari Vishal)

Age (weeks)	Control	T_1 (10x 10^3)
1 week	101.04	102.21
2 weeks	192.50	193.38
3 weeks	301.25	302.67
4 weeks	538.77	471.11
5 weeks	808.81	632.14
6 weeks	1037.84	840.54
7 weeks	1232.41	1026.27
8 weeks	1413.08	1201.66

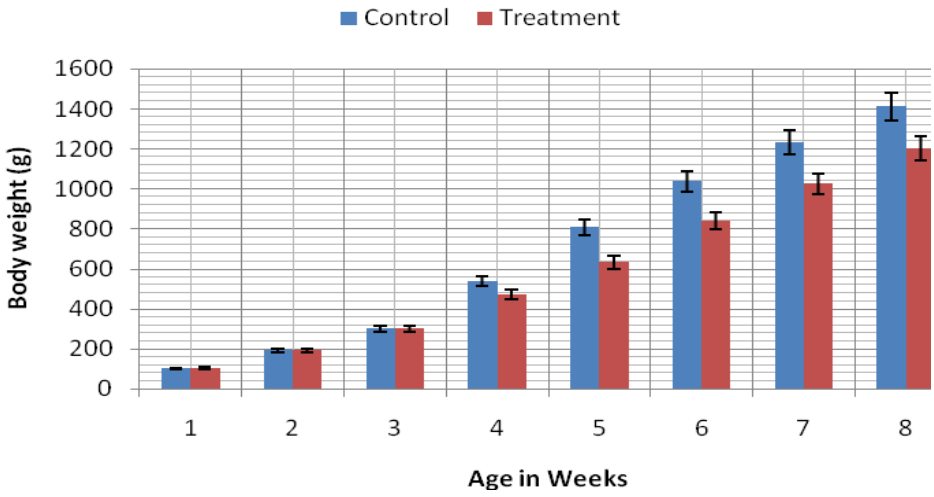


Figure 6: Average weekly body weight (g) in Caribro-91 (Cari Vishal)

Similarly in Caribro-91 (Cari Vishal) birds, the difference in the body weight of birds under control and treatment group upto 3rd week of age was non-significant. However, after infection of *Eimeria tenella* with dose of 10,000 sporulated oocycts, the birds under control group excelled the performance as compared to treatment group from 4 to 8 week of age (Figure 6). The average body weight in control and treatment groups

of Caribro-91 birds were ranged from 101.04 to 1413.08 g and 102.21 to 1201.66 g, respectively.

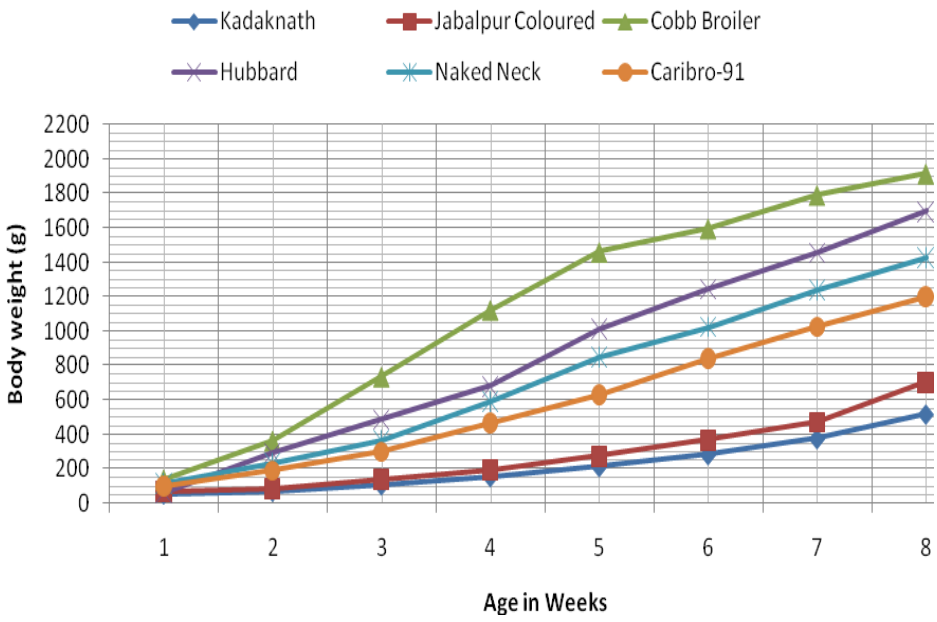


Figure 7. Comparative body weight in different genetic groups

The coccidial infection significantly affected the body mass gain and feed conversion ratio of the birds. The overall comparative body weight in different genetic groups under treatment is shown in the Figure 07.

Lesion Score:

Lesion score was determined as per the method suggested by Johnson and Reid (1970). The caeca from two sacrificed birds from each group were examined microscopically on 4th, 7th and 14th day post infection for observing the condition of the caecal wall, consistency of caecal contents, presence of haemorrhage in the caeca or on the caecal wall, etc. The lesion score for *Eimeria tenella* was graded as described in Table 08.

The lesion scoring was done on the basis of Caecal wall thickness, consistency of caecal contents, presence of haemorrhage in the caeca or on the caecal wall, etc. Birds slaughtered on days 7 and 14 post infection showed scattered petechiae

haemorrhages on the caecal wall, noticeable clotted and un-clotted blood in the caecal contents and thickening of the caecal wall.

Table 08: Lesion Scoring Method (Johnson and Reid, 1970)

S.No.	Lesion in the caecum	Lesion Score
1.	No gross lesion	0
2.	Very few scattered petechinae on the caecal wall, no thickening of the caecal wall, normal caecal contents	+1
3.	Lesion more numerous with noticeable blood in the caecal Contents, caecal wall somewhat thickened, normal caecal Contents	+2
4.	Large amount of blood or caecal cores in caeca, caecal wall greatly thickened, little, if any, faecal content in the Caeca.	+3
5.	Caecal wall greatly distended with blood or large casseous cores, faecal debris lacking or included in cores.	+4
6.	Dead birds were scored as	+5

The lesion score ranged from +1 to +4 among the birds/groups under study. In Jabalpur dual coloured and Kadaknath it ranged from +1 to +2, while Naked neck and Hubbard showed +1 to +3. Caribro-91 and Cobb had the lesion score upto +4. The finding of the lesion score revealed that Kadaknath and Jabalpur dual coloured found to be more resistant to coccidial infection amongst the different genetic groups, while Caribro-91 and Cobb were most susceptible genetic groups. The genetic group involving Naked neck and Hubbard falled in intermediate range of susceptibility.

Oocyst Index

Oocyst index was determined by microscopic examination of mucosal scrapings from the caeca on day 4, 7 and 14 post infection as per the method of Hilbrich (1978) with some modifications. The caecal mucosa was scrapped onto the coverslip and the coverslip was then pressed on a microscopic slide in such a way that the mucosal material spreaded underneath the whole surface area of the coverslip. Five fields of coverslip were viewed for each scrapping i.e. four corners and a central field and the number of oocyst were counted in each field. The oocyst index was graded according to the number of oocysts per field as given in Table 09.

Table 09: Grading of oocyst index

S.No.	Number of oocysts per field	Oocyst index
1	0	0
2	1-20	1
3	20-50	2
4	50-100	3
5	100-200	4
6	>200	5

Oocyst index were determined by microscopic examination of mucosal scrapings from the caeca on 4^{th} to 14^{th} day post infection as per the method of Hilbrich (1978). The oocysts index varied from 1 to 3. The number of oocysts per field ranged from 15 to 100. Oocyst index in Jabalpur dual coloured, Kadaknath and Hubbard were in the range of 1 to 2, while those of Naked neck, Caribro-91 and Cobb were 1 to 3.

Oocysts production (OPG)

Oocyst production was determined in terms of oocysts per gram (OPG) to see oocysts production in experimental birds infected with *E. tenella*. The faecal samples of remaining birds from each group were collected, separately, from day 5 to day 9 post infection and the OPG of faeces in each was estimated by following the method of Davies *et al*. (1963), using McMaster counting chamber. The faeces voided by the broiler chicks was collected daily and was taken to the laboratory for estimation of OPG. Three grams of faeces was homogenized in 42 ml of tap water and strained through 100 mesh metallic sieve layered with muslin cloth and aliquot of 15 ml was then taken in a graduated centrifuge tube, centrifuged for 10 min at 2000 rpm and the supernatant was discarded. The sediment was resuspended in saturated sodium chloride solution to make the total original volume of 15 ml in which the total number of oocysts was counted after suitably diluting this suspension. Then both side of McMaster counting chambers were carefully loaded to avoid producing bubbles, each chamber holds about 0.15 ml of slurry. Preparation was left to stand for a minimum of five minutes. The oocysts in both sides of chamber were counted under the 10 x magnification. Eggs outside the grid were not counted and OPG was calculated by the following formula.

OPG = (No. of oocysts in side 1 + No. of oocysts in side 2) X 50.

Average OPG count in different chicken breed/lines:

The faecal samples of Cobb chickens from each group were collected, separately, from day 5 to day 10 post infection and the OPG (oocyst per gram) of broiler chicks in each group was estimated following the method of Davies *et al*. (1963), using a McMaster counting chamber. The average oocysts per gram (OPG) in different genetic groups under investigation examined, separately, from day 5 to day 10 post infection is given below in **Table 10**. The shedding of oocysts was recorded as early as on the 5th day post challenge. The oocysts number shed varied at different stages of challenge. The oocysts production was maximum on day 7th and then the OPG started to decline upto day 10 post challenges (Figure 8).

Table 10. Average OPG count in different genetic groups

Breed/ Days	Kadaknath	JBP Coloured	Cobb	Hubbard	Caribro-91	Naked Neck
5th day	3576.31	3591.66	11350.00	8222.01	6419.44	5194.40
6th day	6465.78	6465.78	14483.00	11297.00	10091.69	8158.00
7th day	6384.21	9260.00	18808.00	16011.00	12188.89	11956.00
8th day	3468.42	6384.21	10879.00	11458.00	7963.88	8211.00
9th day	3576.31	3468.42	5497.20	5894.40	4272.22	4666.00
10th day	2218.56	2218.56	3461.20	3216.00	1802.96	2602.00

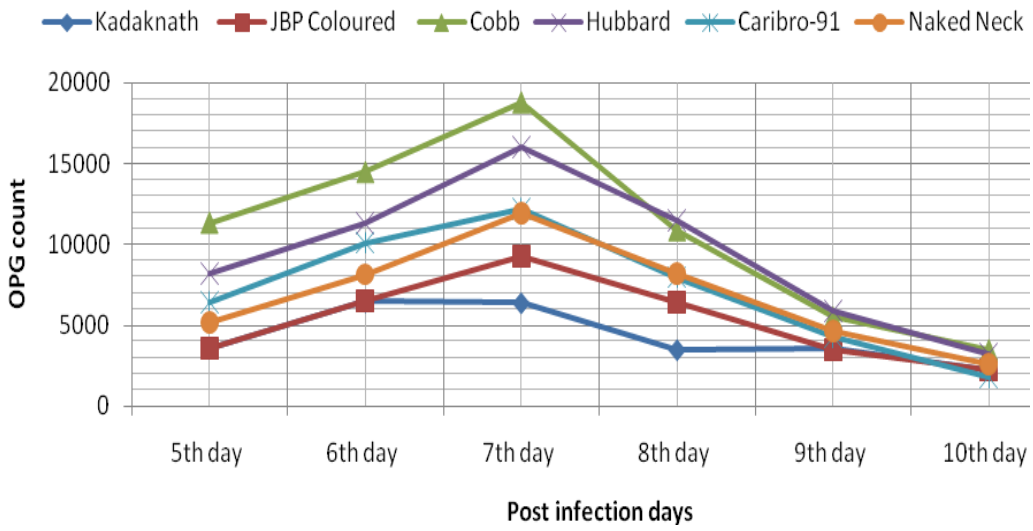


Figure 8. Average OPG in different genetic groups

The least squares analysis of variance employed to find out the association of breed, dose and breed x dose interaction on OPG have been presented in Table 11. The results revealed significant effect (P<0.01) of breeds and dosage of coccidial challenge at day 5 to 9 post infection. The effect of breed x dose interaction was found to be significant on day 6 post challenge while for remaining day post infection, it was non significant.

Table 11. Analysis of variance of breed, coccidial dose and their interaction effect on OPG at day 5 to 9 post infection.

Interval	Source of variation	Breed	Dose	Breed X Dose	Error
Day 5	**MS**	202308356.0 (2)	42187500.0 (1)	1091458.0 (2)	3091517.0 (102)
	F-value	65.44**	13.65**	0.35	-
Day 6	**MS**	287367523.0 (2)	55541690.0 (1)	19174329.0 (2)	4535227.0 (102)
	F-value	63.36**	12.25**	4.24*	-
Day 7	**MS**	375979884.0 (2)	76087245.0 (1)	6415995.0 (2)	4955295.0 (102)
	F-value	75.87**	15.35**	1.29	-
Day 8	**MS**	299780486.0 (2)	27200370.0 (1)	8670440.0 (2)	3442200.0 (102)
	F-value	87.09**	7.90**	2.52	-
Day 9	**MS**	80841944.0 (2)	23660208.0 (1)	2940833.0 (2)	1419623.0 (102)
	F-value	56.95**	16.67**	2.07	-

** Significant (p<0.05),** Significant (p<0.01), MS -Mean Sum of squares, Values in parentheses are degree of freedom*

Mortality

All the experimental groups were also observed for mortality of birds, if any, during the study and it was expressed in percentage for that group. The Mortality amongst the different genetic groups ranged from 0 to 15 %. No mortality was observed in Kadaknath and Jabalpur dual coloured birds, while maximum mortality was recorded in Caribro-91 (15%).

MAJOR HISTOCOMPATIBILTY COMPLEX (MHC) GENE POLYMORPHISM

A pair of primers specific to MHC B-Lβ (class II) as given below was custom synthesized at Integrated DNA Technology (IDT) for first round of amplification (Zheng *et al*., 1999).

B-LβII-F 5' – CG TTC TTC TTC TRC GGT RBG AT – 3'

B-LβII-R 5' – TA GTT GTG CCG GCA GAM CSY G – 3'

Where, R = A or G, M = A or C, S = G or C, Y = C or T, B = G, C or T.

For PCR-SSP, five pairs of chicken MHC B-Lβ (class II) family haplotype specific primers (Zheng *et al*., 1999) were custom synthesized at Integrated DNA Technology (IDT) for haplotyping of chicken. Primers supplied in the translucent form were dissolved in sterile nuclease free water and was diluted to give a concentration of 10 pmol/µl with sterile nuclease free water.

The B-LβII family specific PCR products were diluted 1:100 times and 1 µl of dilution were subjected to PCR-SSP with following standardized cycling condition (Table 12). To confirm the targeted PCR amplification, 5µl of PCR product from each tube was mixed with 1µl of 6X loading dye buffer and was visualized as a single compact band by UV transilluminator and photographed (Gel documentation system, Bio-Rad, USA).

Table 12. PCR -SSP cycling Profile

Target Haplotype	up-primer	dn-primer	Annealing T (^{o}C)	Cycles	Product size (bp)
B2	4up8	1DN69	55	18	222
B13	1up32	1DN65	60	15	141
B15	3up8	2DN69	50	18	222
B19	2up8	1DN66	55	15	213
B21	1up8	2DN66	55	18	213

MHC genotyping of birds under different genetic groups:

Genotyping was carried out using PCR-SSP (Polymerase Chain Reaction with Sequence Specific Primers). A 235bp, exon-2 region of chicken MHC BL-β II family gene was amplified using a set of five primers. B2 (222bp), B13 (141bp), B15 (222bp), B19 (213bp) and B21 (213bp). Genotyping by PCR-SSP revealed a total of 15 genotypes in the studied sample size of experimental birds. Allelic and genotypic

frequencies were estimated using POPGENE 32(version1.32), microsoft Windows-based freeware for population genetic analysis and the population was tested for genetic equilibrium at this locus. The allelic and genotypic frequencies at MHC BL-β II family gene in different genetic groups of chicken have been presented in Tables 13 and 14.

Kadaknath genetic group revealed 8 genotypes in the population (Figure 9). The genotypic frequencies for observed genotypes B_2B_2, B_2B_{13}, B_2B_{15}, B_2B_{19}, $B_{13}B_{13}$, $B_{13}B_{19}$, $B_{15}B_{19}$ and $B_{19}B_{19}$ were 0.167, 0.300, 0.183, 0.100, 0.083, 0.067, 0.067 and 0.033, respectively. The highest genotypic frequency was observed for B_2B_{13} (0.300) and lowest for $B_{19}B_{19}$ (0.033) genotypes in studied Kadaknath population.

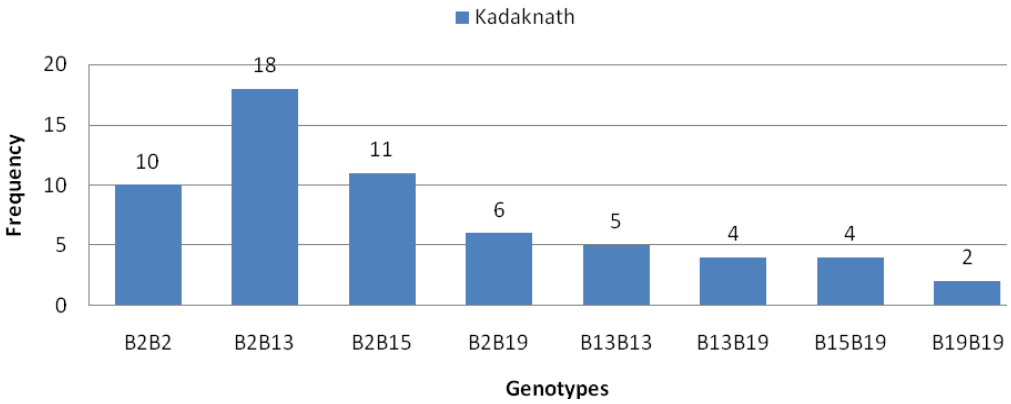


Figure 9. Observed genotypes/haplotypes in Kadaknath chcicken.

In **Caribro-91 (Cari Vishal)**, 10 genotypes were encountered with B15B19 (12) leading the tally amongst B2B13 (4), B13B13 (3), B13B15 (6), B13B19 (2), B15B15 (9), B15B21 (2), B19B19 (11), B19B21 (7) and B21B21 (4). The different genotypes / haplotypes are shown in Figure 10.

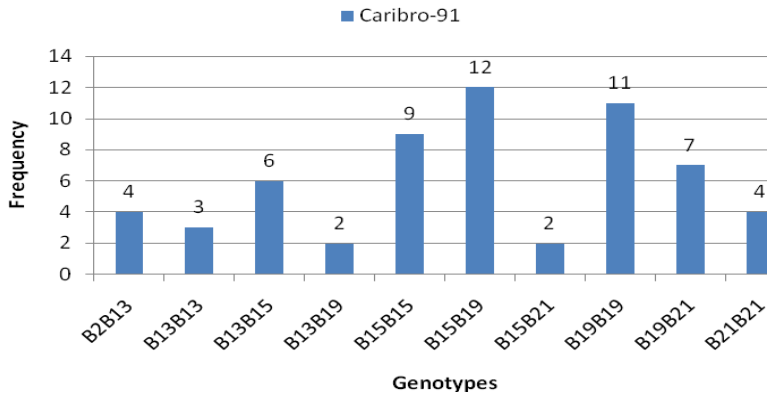


Figure 10. Observed genotypes/haplotypes in Caribro-91 broiler line.

The numbers of genotype found in the **Cobb** strain were 9. In this experimental flock B19B19 genotype was the highest in number i.e. 15 followed by B2B2, B2B19, B2B13, B2B15, B2B21, B13B15, B19B21 and B21B21 (Figure 11). Genotype B19B19 was found predominantly in both the flock of Caribro-91 and Cobb broiler birds.

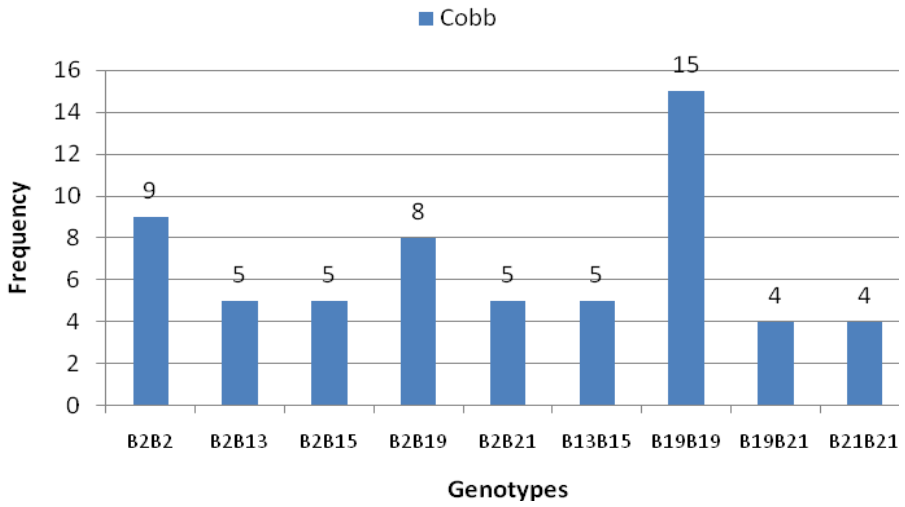


Figure 11. Observed genotypes/haplotypes in Cobb broilers.

In **Hubbard broiler**, 8 genotypes were found. B2B19, B15B19 and B2B15 were predominantly observed with the respective frequency of 15, 14 and 11. The other genotypes viz. B2B2, B19B21, B13B19, B15B15 and B21B21 were having 6, 5, 4, 4 and 1 numbers (Figure 12).

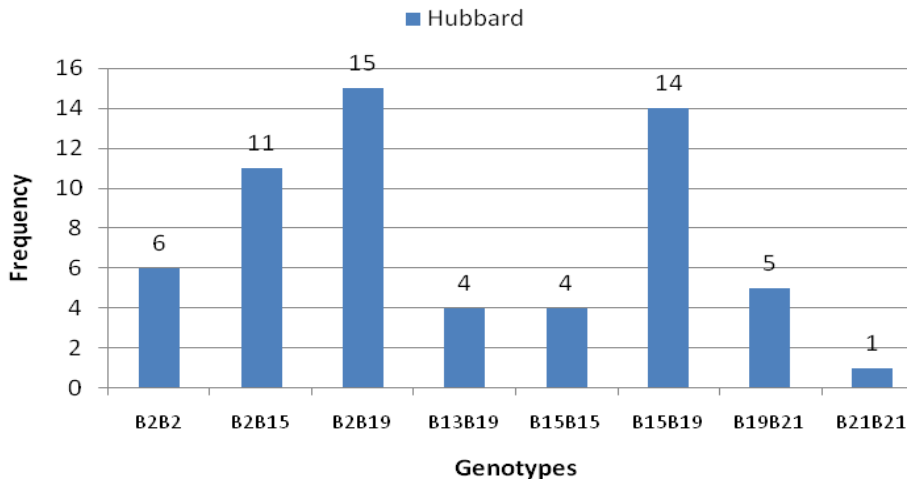


Figure 12. Observed genotypes/haplotypes in Hubbard broilers.

Naked neck and **Jabalpur dual coloured** were found to have 10 genotypes each. In naked neck genotypes with highest frequencies were B15B19 and B19B19 with 13 each in number (Figure 13).

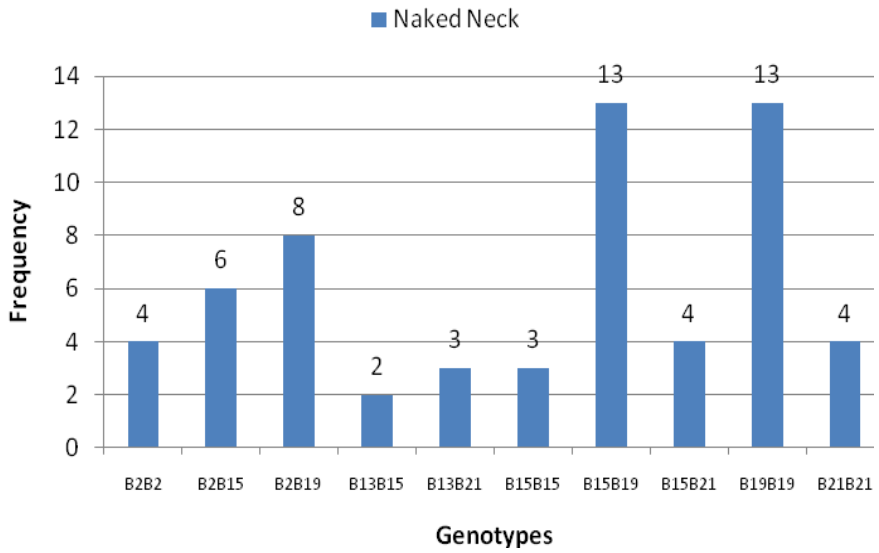


Figure 13. Observed genotypes/haplotypes in Naked Neck broiler line.

The genotype B2B13 was highest in Jabalpur dual coloured with frequency 13 followed by B2B19 with frequency of 11 (Figure 14).

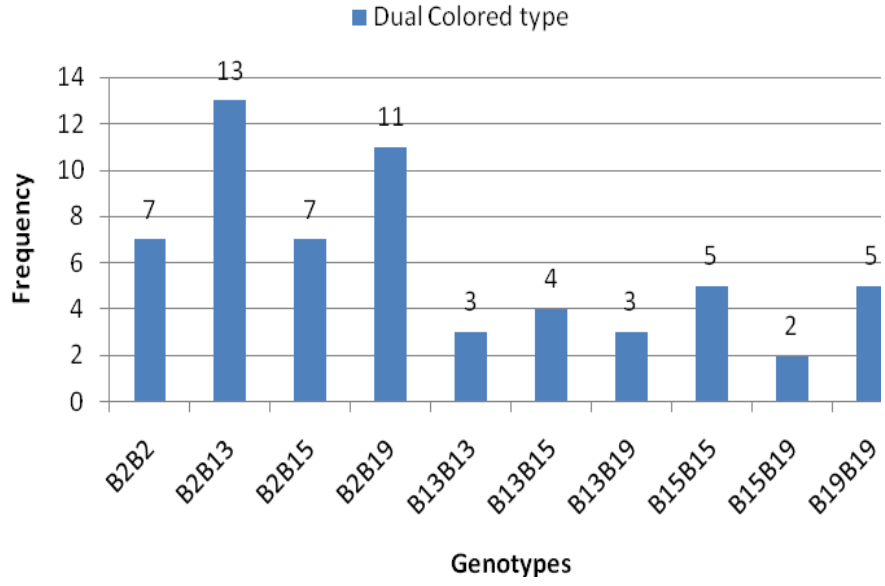


Figure 14. Observed genotypes/haplotypes in Jabalpur dual coloured.

Table 13: Allelic frequency at MHC B-L β II family gene locus in different genetic groups of chicken

Allele	Allelic frequency					
	Kadaknath	Dual Coloured type	Cobb	Hubbard	Caribro-91	Naked Neck
B2	0.458	0.375	0.342	0.317	0.033	0.183
B13	0.267	0.217	0.083	0.033	0.150	0.042
B15	0.125	0.192	0.083	0.275	0.317	0.258
B19	0.150	0.217	0.342	0.317	0.358	0.392
B21	0.000	0.000	0.150	0.058	0.142	0.125

Table 14: Genotypic frequency at MHC B-L β II family gene locus in different genetic groups of chicken

S. No	Genotypes	Genotypic frequency					
		Kadaknath	Caribro-91	Cobb	Hubbard	Naked Neck	Jabalpur dual coloured
1.	B2B2	0.167	0.000	0.150	0.100	0.067	0.117
2.	B2B13	0.300	0.067	0.083	0.000	0.000	0.217
3.	B2B15	0.183	0.000	0.083	0.183	0.100	0.117
4.	B2B19	0.100	0.000	0.133	0.250	0.133	0.183
5.	B2B21	0.000	0.000	0.083	0.000	0.000	0.000
6.	B13B13	0.083	0.050	0.000	0.000	0.000	0.050
7.	B13B15	0.000	0.100	0.083	0.000	0.033	0.067
8.	B13B19	0.067	0.033	0.000	0.067	0.000	0.050
9.	B13B21	0.000	0.000	0.000	0.000	0.050	0.000
10.	B15B15	0.000	0.150	0.000	0.067	0.050	0.083
11.	B15B19	0.067	0.200	0.000	0.233	0.217	0.033
12.	B15B21	0.000	0.033	0.000	0.000	0.067	0.000
13.	B19B19	0.033	0.183	0.250	0.000	0.217	0.083
14.	B19B21	0.000	0.117	0.067	0.083	0.067	0.000
15.	B21B21	0.000	0.067	0.067	0.017	0.000	0.000
χ^2 value		11.167^{NS}	43.180*	60.773*	27.702*	36.179*	10.792^{NS}

NS- Non Significant, ** highly significant (P<0.01), Figures in parenthesis indicates the numbers.

Association of MHC gene polymorphic variants with lesion score, oocyst index and OPG at day 4, 7 and 14 post challenge

The caeca of the slaughtered birds were removed from two chickens, under each group at day 4, 7 and 14 post infections and used for lesion scoring as per standard procedure (Table 8). The oocyst index was determined by microscopic examination from each segment of caeca of birds sacrificed for lesion score at day 4, 7 and 14 post inoculation for counting of oocysts per field as per the standard method (Table 9). The analysis of variance was conducted to find out the effect of breed, interval, dosage of coccidial challenge and their interactions on lesion score, oocyst index and OPG on days 4, 7 and 14 post challenges in slaughtered birds from each genetic group (Table 15).

Table 15. Analysis of variance for lesion score, Oocyst index and OPG count

Source of Variation	lesion score		Oocyst index		OPG	
	MS	F- value	MS	F- value	MS	F- value
Breed	2.7222 (2)	11.31**	0.9630 (2)	6.50**	18390691.00 (2)	157.70**
Interval	3.1667 (2)	13.15**	4.7963 (2)	32.38**	330609611.00 (2)	2834.93**
Dose	31.5000 (2)	130.85**	12.0741 (2)	81.50**	142547758.00 (2)	1222.33**
Breed x Interval	0.2222(4)	0.92	0.2130 (4)	1.44	15159112.00 (4)	129.99**
Breed x Dose	0.9722 (4)	4.04**	0.2407 (4)	1.62	5627892.00 (4)	48.26**
Interval X Dose	0.8333 (4)	3.46**	1.2407 (4)	8.38**	87756290.00 (4)	752.50**
Breed X Interval x Dose	0.2639 (8)	1.10	0.1574 (8)	1.06	4553227.00 (8)	40.76**
Error	0.2407 (27)		0.1481 (27)		116620.00 (27)	

** Significant ($p<0.01$), Values within parentheses are degree of freedom

Effect of breeds, dosage, interval and their interactions on lesion score

Results of analysis of variance revealed highly significant effect ($p<0.01$) of breed, interval, dose, breed x dose interaction and interval x dose interaction and non significant effect of breed x interval interaction and breed x interval x dose interaction on lesion score in all six genetic groups (Table 12). The different and descriptive caecal lesions observed in *Eimeria tenella* infection have been presented in Plate 3.

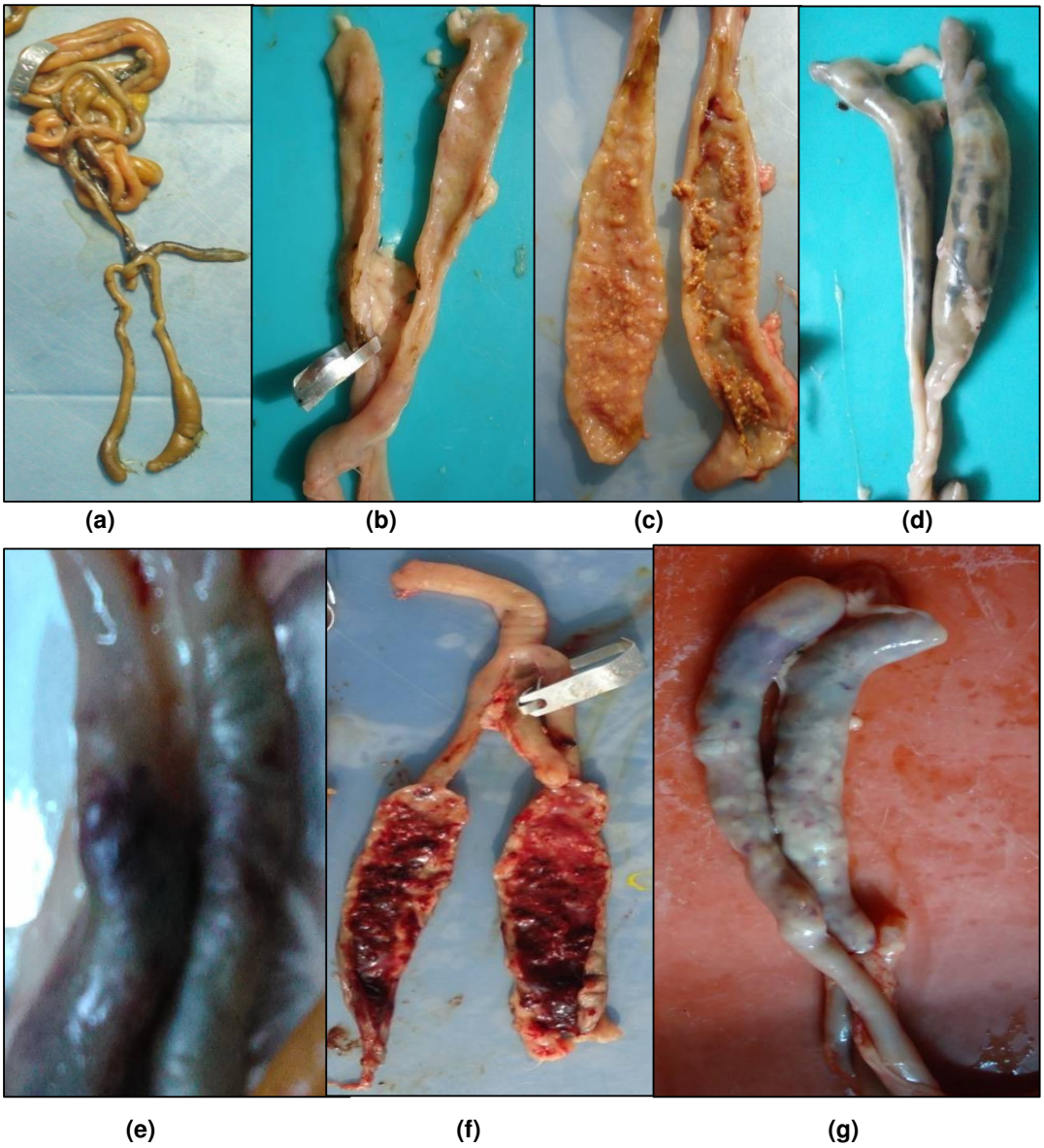


Plate 3: Descriptive caecal lesions in *Eimeria tenella* infection

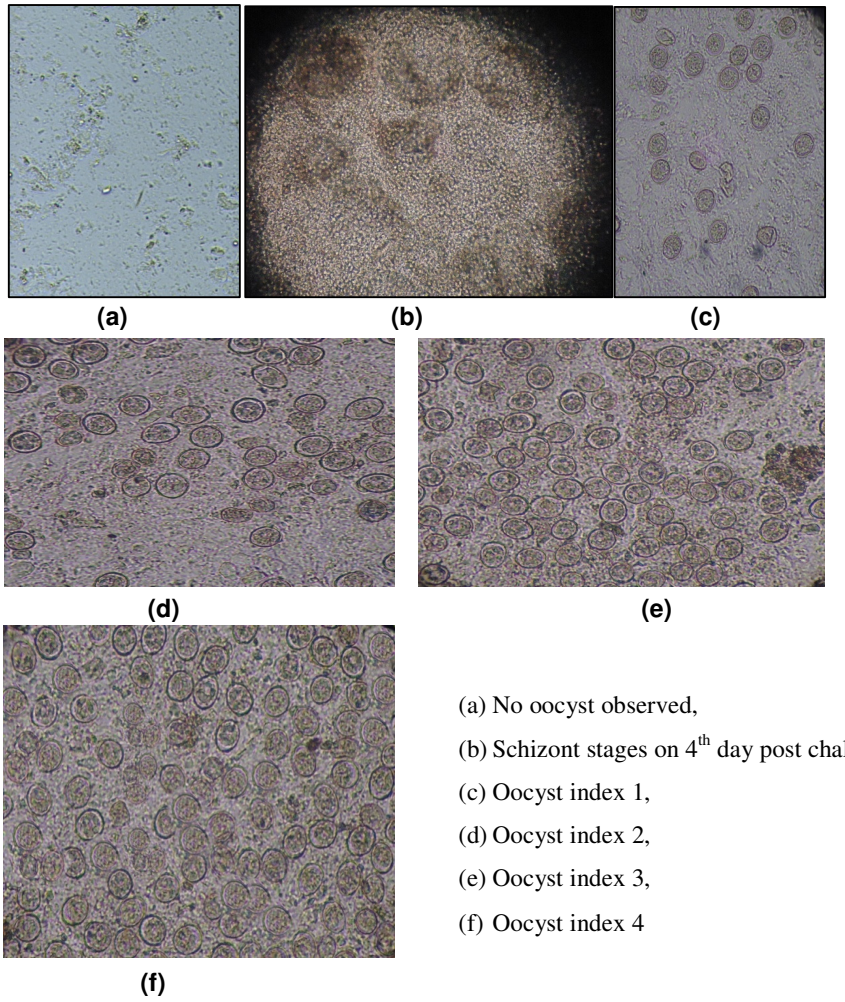
(a) Normal caeca of birds with no gross lesions,(b) Scattered petechiae on the caecal wall, (c) Numerous lesions with noticeable blood in caecal contents, (d) Ballooning of caecal loop with caecal core, (e) Large amount of blood or caecal cores in caeca (f) More numerous and haemorrhagic blood in caecal contents (g) Distended caecum with haemorrhage and packed with casseous core.

The mean lesion scores in Kadaknath, Jabalpur dual Coloured, Naked Neck, Caribro-91, Hubbard and Cobb birds have been presented in Table 16. The mean lesion score showed increasing trend till day 7 post challenge and then declined at day 14 post challenge. The highest mean lesion score on day 7 post challenge was recorded in Cobb (3.25±0.25), followed by Hubbard (2.75±0.29), Caribro-91 (2.75±0.25), Naked Neck (2.50±0.29), Jabalpur dual Coloured (1.75±0.25) and Kadaknath (1.50±0.29). On day 14 post challenge when mean lesion score was minimum in all the six genetic groups, the

lowest mean value among all the genetic groups was recorded for Kadaknath (0.75±0.25) birds. These results indicated that Cobb genetic group was more susceptible for coccidial infection as compared to other five genetic groups, whereas Kadaknath group was most resistant to coccidial infection.

Table 16. Average (±SE) of lesion score at day 4, 7 and day 14 in challenged birds of in different genetic groups

Interval	Kadaknath	Jabalpur Dual Coloured	Naked Neck	Caribro-91	Hubbard	Cobb
Day 4	1.00±0.00	1.25±.0.00	1.50±0.29	1.50±0.25	1.50±0.25	2.00±0.00
Day 7	1.50±0.29	1.75±0.25	2.50±0.29	2.75±0.25	2.75±0.29	3.25±0.25
Day 14	0.75±0.25	1.00±0.00	1.25±0.25	1.25±0.25	1.25±0.29	1.50±0.29



(a) No oocyst observed,
(b) Schizont stages on 4th day post challenge,
(c) Oocyst index 1,
(d) Oocyst index 2,
(e) Oocyst index 3,
(f) Oocyst index 4

Plate 4: Different oocyst indices (OI) observed in *E. tenella* infection

Effect of breeds, dosage, interval and their interactions on oocyst index

The result of analysis of variance for oocyst index indicated highly significant effect ($P<0.01$) of breed, interval, dose and interval x dose interaction and non significant effect ($P>0.05$) of breed x interval interaction, breed x dose interaction and breed x interval x dose interaction (Table 11). The mean oocysts indexes for all six genetic groups have been presented in Table 17. The different oocyst indices (OI) observed in *Eimeria tenella* infection have been presented in Plate 4.

Table 17. Average (±SE) of oocysts index at day 4, 7 and day 14 in different genetic groups

Interval	Kadaknath	Jabalpur Dual Coloured	Naked Neck	Hubbard	Caribro-91	Cobb
Day 4	1.00±0.00	1.00±0.00	1.25±0.25	1.5 0±0.25	1.50±0.29	2.00±0.00
Day 7	1.00±0.25	1.50±0.25	2.25±0.48	2.50±0.25	2.50±0.29	2.75±0.29
Day 14	0.50±0.29	1.00±0.25	1.25±0.29	1.25±0.00	1.75±0.25	1.50±0.25

Oocyst index was maximum on days 7 post challenge and minimum on day 14 post challenge in all the six genetic groups. The highest mean oocysts index was found in Cobb (2.75±0.29) as compared to other five genetic groups on day 7 post challenge. The lowest mean oocysts index was observed in Kadaknath (0.50±0.29) on day 14 post challenge. On comparison, the Cobb genetic group was more susceptible for coccidial infection than other five genetic groups, whereas Kadaknath was found to be most resistant to coccidial infection.

Association of cytokine genes expression level in birds

The association between cytokine genes expression level in relation to coccidial resistance in different breeds/lines at different intervals (4, 7 14 days post infection) was studied using real-time PCR. The expression level of mRNA encoding cytokines genes quantified in intestinal lymphocytes following *E. tenella* infections were upregulated at different intervals of infection showed significant variation in level of cytokine genes. The relative mean fold expression for IL-3 gene ranged from 4.94± 0.37 for dose 1 at day 14 post challenge in Naked Neck to 147.03±0.54 in Hubbard at day 4 post challenge. The highest and lowest relative mean fold expression for IL-10 gene was observed to be 150.64± 0.26 at day 7 post challenge in Naked Neck and 3.06±0.04 in

Jabalpur Dual Coloured at day 14 post coccidial challenge, respectively. For IL-12 gene, the lowest and highest relative means fold expression was observed to be 3.51± 0.26 and 58.89 ± 0.52 , respectively in Naked Neck. The relative mean fold expression for GM-CSF gene was found to be minimum with a value of 7.26±0.08 in Jabalpur Dual Coloured and maximum was seen in Hubbard (275.32±0.44) at day 4 post infection. For TGF- β4 gene, the lowest relative mean fold expression was observed in Naked Neck (11.83±0.60) 2 day 14, while the highest value was recorded in Hubbard (80.44±0.40) at day 4 post coccidial challenge. The relative mean fold expression of IL-1β gene ranged from 9.91±0.14 (Kadaknath) to 105.78±0.52 (Cobb) and 14.47±0.28 (Kadaknath) to 119.01±0.29 (Caribro-91), respectively. The highest and lowest relative mean fold expression for IL-2 gene was found to be 242.19±0.40 and 6.25±0.03 in Cobb at days 4 and 14 post challenge, respectively. However, the relative mean fold expression of IL-2 gene for T_1 was found to be maximum in Cobb (75.84±0.58) and minimum in Kadaknath (12.77±0.38) at day 14 and day 7 post challenge, respectively. For IL-6 gene, the lowest relative mean fold expression was observed in Kadaknath (8.11±0.05) at day 14 while the highest value was recorded in Cobb (99.04±0.92) at day 7 post infection. The relative mean fold expression for IL-17 gene was found to be lowest with a value of 11.04±0.29 in Kadaknath and maximum was seen in Cobb *i.e.,* 197.40±0.37. For IFN-Y gene, the lowest relative mean fold expression was found to be 8.11±0.19 in Kadaknath while the highest value was recorded in Cobb (84.74±0.25) at day 7 post challenge. All the ten cytokine genes showed relative up-regulation in the six genetic groups in responses to coccidiosis

Phylogenetic analysis

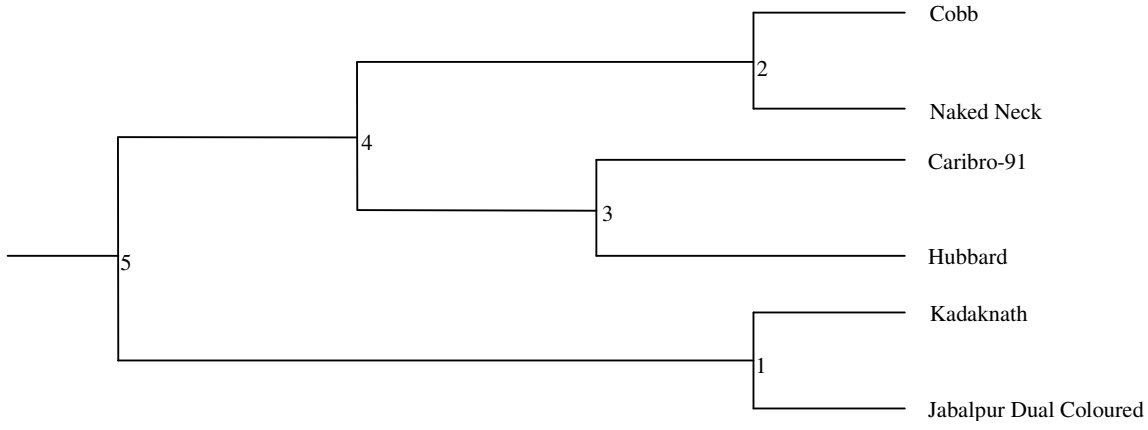


Figure 15: Neighbor joining tree for exon 2 MHC BLβ II gene in six genetic groups.

In order to study the genetic distance among the six genetic groups using sequenced data, the phylogenetic analysis using the software *DNAstar 2.0 MegAlign* by using Clustal x Method was performed. The cluster group thus obtained has been presented in figure 15. The phylogenetic tree showed that the six breeds/lines were divided into two clusters. The first cluster consisted of Cobb, Naked Neck, Caribro-91 and Hubbard. Kadaknath and Jabalpur dual coloured chickens were grouped under second cluster. Kadaknath is more closely related to Jabalpur dual coloured chickens as compared to other genetic groups.

Antibody response to SRBC:

Twelve birds of each identified commercial lines and Kadaknath were kept in separate block. Pre-immune blood samples (0 day) were collected from all birds. Each bird was injected i/m at 2 sites in the breast with 0.5ml/site of a 25% SRBC suspension in PBS at 4^{th} weeks of age. Chicks were received a booster on Day 14 PPI (i.e. at 6^{th} weeks of age). Blood samples were collected on day 4, 7, 14 & 21 of post primary immunization (PPI). All blood samples (2 to 3 ml) were analyzed for anti-SRBC HA test. All blood samples (2 to 3 mL) were allowed to clot overnight at 4 ^{0}C and sera were harvested by centrifugation, heat-inactivated at 56 ^{0}C for 30 min. and then stored at –20 C until assayed. Total anti-SRBC haemagglutinating antibodies were measured in 96-well U-bottomed microtiter plates. An equal volume of 25% SRBC suspension in PBS was added to each serum dilution (final volume of 50 μL), samples were mixed, and then they were allowed to react at room temperature for 2 hr. The haemagglutination titer for a serum was the reciprocal, expressed as $\log_2$ of the highest serum dilution that gave 100% agglutination.

Pre-immune sera (Day 0) from all birds were negative against SRBC in the hemagglutination assay. The effect of identified commercial lines and Kadaknath, age of infection, challenge period (first vs. second) and their interactions were significant. After the first infection, the number of oocysts shed varied among the broiler lines; however, the line variation was dependent on the age of infection.

The Cobb broiler and Caribro-91 (Cari Vishal) birds showed the poorest performance in antibody response to SRBC and challenge with Coccidiosis. These two breed had low antibody titre to SRBC as well as an increased susceptibility to Coccidiosis as compared to other chicken breed/lines. The Naked Neck broiler and Hubbard broilers were comparable in their responses to SRBC and coccidial challenges.

The Kadaknath and Jabalpur dual coloured birds showed maximum response to SRBC as well as minimum lesion scores and oocyst Index as compared to other breeds/lines. These two breed were comparatively more resistance to coccidial challenges.

Summary and conclusions of the project:

The present work was conducted on sixty birds each of Kadaknath, Cobb, Caribro-91, Naked Neck, Hubbard and Jabalpur Dual Coloured chicken reared under the research project funded by Madhya Pradesh Biotechnology Council, Bhopal in Department of Animal Genetics and Breeding, College of Veterinary Science and Animal Husbandry, Jabalpur. Genomic DNA with optimum quality and quantity was used for first round of PCR amplification of exon 2 of MHC B-L β II family gene using a set of degenerating primers. An amplified PCR product of 235 bp size was observed in Kadaknath, Cobb, Caribro-91, Hubbard, Jabalpur Dual Coloured and Naked Neck genetic groups. The amplicons of 222 bp, 141 bp, 222 bp, 213 bp and 213 bp sizes were obtained after secondary PCR amplification of targeted haplotypes i.e. B_2, B_{13}, B_{15}, B_{19} and B_{21}, respectively. The present findings revealed that the MHC locus is highly polymorphic in all the six genetic groups under study. This MHC gene polymorphism can be used in selection for disease resistant traits in these broiler lines in future. A total of 14 genotypes were found in these six genetic groups. The genotype B_2B_{21} was absent in all the genetic groups under study.

The least squares analysis of variance applied to find out the significance of association of breed, polymorphic variants of MHC gene on dosage of coccidial challenge revealed highly significant effect of breed ($p<0.01$) and dosage of coccidial challenge at each stage of post coccidial infection. The effect of genotype on oocysts per gram (OPG) was found to be non-significant. The mean OPG was found to be highest in Cobb and lowest in Kadaknath. The highest OPG count was observed on day 7 post challenge under each dosage of coccidial infection. The analysis of data recorded on day 4 day 7 and day 14 post challenge for lesion score, oocysts index and OPG were done by least squares analysis. Results revealed significant effect ($p<0.01$) of breed, interval, dose and interval x dose interaction on lesion score and oocysts index. The lesion score, oocyst index and OPG count were highest in Cobb and lowest in Kadaknath. The mean lesion score, oocysts index and OPG showed increasing trend upto second interval i.e. on

day 7 post challenge and on third interval (on day 14 post challenge), it was lowest in all the genetic groups.

On comparison with the findings of present investigation, Kadaknath breed showed comparatively less OPG count, lesion score and oocyst index during post coccidial infection. The oocyst index and lesion scoring are often considered as the most important indicators for coccidiosis evaluation, it can be realized that in the similar rearing conditions the economic losses due to coccidiosis can be substantially reduced by selecting Kadaknath birds in poultry rearing practices.

From the results obtained in the present study it can be concluded that:

i. Allelic frequencies of B_2 and B_{13} were found to be highest in Kadaknath while frequencies of B_{15} and B_{19} alleles were highest in Caribro-91. B_{21} allele was absent in Kadaknath and Jabalpur dual coloured birds.

ii. A total of 15 genotypes were observed in the entire population under study. The number of genotypes found in Cobb, Kadaknath and Caribro-91, Naked Neck, Hubbard and Jabalpur Dual Coloured were 9, 8, 10, 10, 8 and 10, respectively. The genotype B13B21 was absent in all the genetic groups.

iii. Kadaknath and Jabalpur dual coloured populations were in Hardy-Weinberg equilibrium while Cobb, Hubbard, Naked Neck and Caribro-91 were not in Hardy-Weinberg equilibrium at this MHC locus. The Kadaknath breed showed maximum variation in region of MHC gene under study.

iv. The birds with genotype B_2B_{21} and $B_{13}B_{19}$ gained higher body weight and also least affected by coccidial infection in comparison to other genotypes among the entire six genetic group.

v. The lowest mean for lesion score, oocyst index and OPG were found in Kadaknath as compared to Jabalpur dual coloured, Cobb, Hubbard, Naked Neck and Caribro-91 genetic groups.

vi. The relative fold expression of cytokine genes increased with dose and was lowest on day 14 post challenge. The relative expression of these cytokine genes

could play a crucial role by driving higher immune responses in susceptible birds to coccidial infection.

vii. Among the six genetic groups, Kadaknath breed was found to have more genetic divergence indicating that this breed is genetically distinct from the other chicken breed included under study.

viii. The Cobb genetic group was most susceptible whereas Kadaknath genetic group was most resistant to coccidial infection. Therefore, it is suggested that Kadaknath breed may be used in developing new lines/strains in combination with commercial birds for future breeding.

Benefits to be accrued to Madhya Pradesh:

Disease prevention is the word of the moment in any livestock species, and more so in the case of poultry, as it is a very popular and fast growing industry. Any research work is well worth looking into. The present work provides a clue to the resistance capacity of the local Kadaknath birds as compared to the multicross varieties, suggesting for the incorporation of local germplasm into the new varieties.

Characterization of the dynamics of chicken cytokine responses associated with *E. tenella* infection will provide a rational basis for use of cytokines and chemokines as therapeutic agents against coccidiosis and this will lessen the drug-based control measures. A non-drug mediated control strategy using genomics, molecular biology and immunology for other poultry diseases thus can be initiated.

The results of this study will definitely contribute to the further understanding of the genetic and immunological basis of resistance to coccidial infection in poultry. Further the findings suggest that there may be a room for some latitude in utilization of Kadaknath breed of chicken for the development of coccidial resistance commercial lines/strains of birds in future.

The valuable information generated during the course of this research investigation can be utilized in the formulation of suitable breeding strategy for developing genetically stable coccidiosis-resistance chicken, thereby increasing the production efficiency of farmers flock.

Man power under Project:

S. No.	Man power (contractual basis)	No.	Name Skilled Labour and Research Fellow	Duration	
				From	To
1	Skilled labour	01	Smt. Meenakshi Choudahry	01/04/2013	31/08/2013
			Shri Narendra Patel	16/08/2013	31/03/2015
2	Research Fellow	01	Dr. Amit Kumar	05/08/2013	20/03/2015

Ph.D. thesis completed under the project: Two

S. No.	Name of the student	Major Advisor	Discipline	Title of Thesis
1.	Dr.M.S. Thakur	Dr. S.N.S. Parmar	Animal Genetics & Breeding	Major histocompatability complex gene polymorphism and expression of cytokine genes in relation to coccidial resistance in poultry*
			*Kadaknath, Cobb broiler and Caribro-91	
2.	Amit Kumar	Dr. B.C. Sarkhel	Animal Genetics & Breeding	Polymorphism of major histocompatibility complex gene and expression profile of cytokine genes in relation to coccidial resistance in poultry#
			#Hubbard, Dual Colour type and Naked Neck	

Equipment acquired or placed order with actual cost

S.No.	Name of equipment	Actual Cost (Rs.)
1.	Rocker-300 (Roter Spin)	24880.00
2.	Mac Master Chamber	8475.00
3.	Spinex with sapre parts	12377.00
4.	Mini Submarine Electrophoresis unit with spare parts	47641.00
5.	Laboratory Autoclave vertical	57969.00
6.	Drinkers and Feeders with stands	24724.00

Financial Progress (2015-2016):

The allotted budget has been spent under various heads viz. recurring and non-recurring. The detailed expenditure (Rs.) of budget is given in **Annexure I to IV**.

Declaration Certificate for Research Projects

Title of the project: Relationship of MHC genes polymorphism with Coccidial resistance in chicken

Name of the Organization: College of Veterinary Science and A.H., N.D.V.S.U., Jabalpur

Principal Investigator: Dr. Mohan Singh Thakur

1. Certified that the amount of Rs. 12.33 lakhs has been utilized on the project for the purpose for which it was sanctioned.
2. Certified that I have satisfied myself that the conditions on which the grants-in-aid was sanctioned have been duly fulfilled.

(Dr. G. Das)
Co- Principal Investigator

(Dr. M. S. Thakur)
Principal Investigator

Head of the Institution: **Dean**
College of Vety. Sci. & A.H.
NDVSU, Jabalpur Signature

Director of Research Services,
Nanaji Deshmukh Veterinary Science University, Signature
Jabalpur (M.P.)

SUMMARY AND CONCLUSIONS OF THE PROJECT

Title of the project: Relationship of MHC genes polymorphism with Coccidial resistance in chicken
Name of the Organization: College of Veterinary Science and A.H., N.D.V.S.U., Jabalpur
Principal Investigator: Dr. Mohan Singh Thakur

1. SUMMARY

The present investigation was conducted on Kadaknath, Caribro-91(Cari Vishal), Jabalpur Dual Coloured, Hubbard, Naked Neck birds and Cobb broiler chicken under the research project funded by Madhya Pradesh Biotechnology Council, Bhopal, Department of Animal Genetics and Breeding, College of Veterinary Science and Animal Husbandry, Nanaji Deshmukh Veterinary Science University (NDVSU), Jabalpur. Sixty day old broiler chicks each of Kadaknath, Jabalpur Dual Coloured, Hubbard and Cobb broiler chicks procured from the hatchery were used in the study while fertile eggs of Caribro-91(Cari Vishal) and Naked Neck broiler chicken were brought from Central Avian Research Institute, Izzatnagar (U.P.) and were hatched in the Department of Poultry Science, College of Veterinary Science and Animal Husbandry, NDVSU, Jabalpur.

The battery brooder house was fumigated by using a combination of 35 ml of formalin (40 per cent formaldehyde) and 10 g potassium permanganate solution for one cubic meter of space. On arrival (day 1) they were wing-banded, weighed and randomly allocated to the treatments. The birds were maintained in the brooder batteries kept inside a well ventilated room in the college experimental poultry shed unit under proper coccidian free conditions. The usual precautions for raising chicks under coccidian free conditions were observed. The standard chick diet free of any anti-coccidial additives and sanitized tap water were given to the birds *ad lib* throughout the experiment.

The *Eimeria tenella* inoculums were prepared separately in three different stages i.e. harvesting of oocysts, sporulation of oocysts and storage of inoculums. The number of sporulated oocysts in the suspension was estimated and the volume was adjusted to contain the 10,000 sporulated oocysts/ml of suspension using McMaster counting chamber.

The chicks from all the experiment groups were weighed at day old and at weekly interval upto 8 weeks of age. The difference in the body weight of Kadaknath,

Jabalpur colored birds, Cobb, Caribro-91, Hubbard and Naked neck under control and treatment group upto 3rd week of age was non-significant. However, after infection of *Eimeria tenella* with dose of 10,000 sporulated oocycts, the reduction in body weight was observed in treatment groups from 4 to 8 weeks of age in all the six genetic groups.

The average body weight from 1 to 8 weeks of age in control and treatment groups of Kadaknath, Jabalpur dual colored, Cobb, Hubbard, Naked Neck and Caribro-91 (Cari Vishal) birds were ranged from 54.68 to 658.71 g and 53.71 to 518.21 g, 67.61 to 777.58 g and 70.58 to 705.82 g, 134.29 to 2161.43 g and 141.39 to 1914.73 g, 65.41 to 2045.42 g and 63.11 to 1696.57 g, 113.80 to 1624.85 g and 115.24 to 1426.11 g, and 101.04 to 1413.08 g and 102.21 to 1201.66 g, respectively. The coccidial infection significantly affected the body mass gain and feed conversion ratio of the birds.

Lesion score was determined as per the method suggested by Johnson and Reid (1970). The lesion score ranged from +1 to +4 among the birds/groups under study. In Jabalpur dual coloured and Kadaknath it ranged from +1 to +2, while Naked neck and Hubbard showed +1 to +3. Caribro-91 and Cobb had the lesion score upto +4. The finding of the lesion score revealed that Kadaknath and Jabalpur dual coloured found to be more resistant to coccidial infection amongst the different genetic groups, while Caribro-91 and Cobb were most susceptible genetic groups. The genetic group involving Naked Neck and Hubbard was in intermediate range of susceptibility.

Oocyst index were determined by microscopic examination of mucosal scrapings from the caeca on 4th to 14th day post infection as per the method of Hilbrich (1978). The oocysts index varied from 1 to 3. The number of oocysts per field ranged from 15 to 100. Oocyst index in Jabalpur dual coloured, Kadaknath and Hubbard were in the range of 1 to 2, while those of Naked Neck, Caribro-91 and Cobb were 1 to 3.

Oocyst per gram (OPG) of birds from all six genetic groups was estimated using McMaster counting chamber by following the method of Davies *et al*. (1963), from day 5 to 9 post infection. The shedding of oocysts was recorded as early as on the 5th day post challenge. The oocysts number shed varied at different stages of challenge. The oocysts production was highest on day 7th and then the OPG started to decline upto day 10 post challenges.

The Mortality amongst the different genetic groups ranged from 0 to 15 %. No mortality was observed in Kadaknath and Jabalpur dual coloured birds, while maximum mortality was recorded in Caribro-91 (15%).

Genotyping was carried out using PCR-SSP (Polymerase Chain Reaction with Sequence Specific Primers). A 235bp, exon-2 region of chicken MHC BL-β II family gene was amplified using a set of five primers *i.e.,* B2 (222bp), B13 (141bp), B15 (222bp), B19 (213bp) and B21 (213bp). Genotyping by PCR-SSP revealed a total of 15 genotypes in the studied sample size of experimental birds. The number of genotypes found in Cobb, Kadaknath and Caribro-91, Naked Neck, Hubbard and Jabalpur Dual Coloured were 9, 8, 10, 10, 8 and 10, respectively. Allelic frequencies of B_2 and B_{13} were found to be highest in Kadaknath while frequencies of B_{15} and B_{19} alleles were highest in Caribro-91. B_{21} allele was absent in Kadaknath and Jabalpur dual coloured birds.

The mean lesion score showed increasing trend till day 7 post challenge and then declined at day 14 post challenge. The highest mean lesion score on day 7 post challenge was recorded in Cobb (3.25±0.25), followed by Hubbard (2.75±0.29), Caribro-91 (2.75±0.25), Naked Neck (2.50±0.29), Jabalpur dual Coloured (1.75±0.25) and Kadaknath (1.50±0.29). These results indicated that Cobb genetic group was more susceptible for coccidial infection as compared to other five genetic groups, whereas Kadaknath group was most resistant to coccidial infection.

Oocyst index was maximum on days 7 post challenge and minimum on day 14 post challenge in all the six genetic groups. The highest mean oocysts index was found in Cobb as compared to other five genetic groups and the lowest mean oocysts index was observed in Kadaknath. On comparison, the Cobb genetic group was more susceptible for coccidial infection than other five genetic groups, whereas Kadaknath was found to be most resistant to coccidial infection.

The association between cytokine genes expression level in relation to coccidial resistance in different breeds/lines at different intervals (4, 7 14 days post infection) was studied using real-time PCR. The expression level of mRNA encoding cytokines genes quantified in intestinal lymphocytes following *E. tenella* infections were upregulated at different intervals of infection showed significant variation in level of cytokine genes. All the ten cytokine genes showed relative up-regulation in the six genetic groups in responses to coccidiosis

The phylogenetic analysis showed that the six breeds/lines were divided into two clusters. The first cluster consisted of Cobb, Naked Neck, Caribro-91 and Hubbard. Kadaknath and Jabalpur dual coloured chickens were grouped under second cluster.

Kadaknath is more closely related to Jabalpur dual coloured chickens as compared to other genetic groups.

The effect of antibody response to SRBC in five identified commercial lines and Kadaknath with age of infection, challenge period (first vs. second) and their interactions were significant. After the first infection, the number of oocysts shed varied among the broiler lines; however, the line variation was dependent on the age of infection. The Cobb broiler and Caribro-91 (Cari Vishal) birds showed the poorest performance in antibody response to SRBC and challenge with coccidiosis. These two breed had low antibody titre to SRBC as well as an increased susceptibility to coccidiosis as compared to other chicken breed/lines. The Naked Neck broiler and Hubbard broilers were comparable in their responses to SRBC and coccidial challenges. The Kadaknath and Jabalpur dual coloured birds showed maximum response to SRBC as well as minimum lesion scores and oocyst Index as compared to other breeds/lines. These two breed were comparatively more resistance to coccidial challenges.

2. CONCLUSIONS:

From the results obtained in the present study it can be concluded that:

ix. Allelic frequencies of B2 and B13 were found to be highest in Kadaknath while frequencies of B15 and B19 alleles were highest in Caribro-91. B21 allele was absent in Kadaknath and Jabalpur dual coloured birds.

x. A total of 15 genotypes were observed in the entire population under study. The number of genotypes found in Cobb, Kadaknath and Caribro-91, Naked Neck, Hubbard and Jabalpur Dual Coloured were 9, 8, 10, 10, 8 and 10, respectively. The genotype B13B21 was absent in all the genetic groups.

xi. Kadaknath and Jabalpur dual coloured populations were in HW equilibrium while Cobb, Hubbard, Naked Neck and Caribro-91 were not in HW equilibrium at this MHC locus.

xii. The birds with genotype B2B21 and B13B19 gained higher body weight and also least affected by coccidial infection in comparison to other genotypes among the entire six genetic group.

xiii. The relative fold expression of cytokine genes increased with dose and was lowest on day 14 post challenge. The relative expression of these cytokine genes could play a crucial role by driving higher immune responses in susceptible birds to coccidial infection.

xiv. Among the six genetic groups, Kadaknath breed was found to have more genetic divergence indicating that this breed is genetically distinct from the other chicken breed included under study.

xv. The lowest mean for lesion score, oocyst index and OPG were found in Kadaknath as compared to Jabalpur dual coloured, Cobb, Hubbard, Naked Neck and Caribro-91 genetic groups. The Cobb genetic group was most susceptible whereas **Kadaknath genetic group was most resistant to coccidial infection**.

xvi. The present work provides a clue to the resistance capacity of the local Kadaknath birds as compared to the multicross varieties, suggesting for the incorporation of local germplasm into the new varieties.

xvii. These results will definitely contribute to the further understanding of the genetic and immunological basis of resistance to coccidial infection in poultry. **These findings suggest that there may be a room for some latitude in utilization of Kadaknath breed of chicken for the development of coccidial resistance commercial lines/strains of birds in future, thereby increasing the production efficiency of farmers flock.**

INFORMATIONS OF PROJECT

"RELATIONSHIP OF MHC GENES POLYMORPHISM WITH COCCIDIAL RESISTANCE IN CHICKEN"

1. List of Publications (with impact factor/NAAS Rating):

• Research Articles Published:

- ✓ Thakur, M.S., Parmar, S.N.S., Amit Kumar, Tomar, S.S., Sarkhel, B.C., Das. G and Shrivastav. A.B. (2015). Evaluation of Kadaknath chicken for coccidial resistance by oocyst count, lesion scoring and oocyst index in *Eimeria tenella* infection. ***Journal of Animal Research***, **5(3):** 579-583.
  NAAS Rating: ***4.49***
- ✓ Thakur, M.S., Parmar, S.N.S., Amit Kumar and Tomar, S.S. (2015). Sequence specific polymorphism of exon-2 MHC B-Lβ II family gene in cobb broiler of chicken. ***Journal of Interacademicia***, **19 (3)**: 413-416.
  NAAS Rating: ***2.34***

• Research Articles Accepted:

- Thakur, M.S., Parmar, S.N.S., Amit Kumar (**2015**). Genetic polymorphism at exon-2 MHC B-LβII family gene by PCR-SSP in Kadaknath chicken. ***Indian Journal of Biotechnology***. (**Accepted**).
  NAAS Rating: ***6.39***
- Thakur, M.S., Parmar, S.N.S., Amit Kumar and Tomar, S.S. (**2016**). Molecular characterization of B-Lβ II family alleles in Caribro-Vishal chicken by PCR-SSP. ***Indian Journal of Field Veterinarians***. (**Accepted**).
  NAAS Rating: ***3.69***
- Kumar, Amit, Sarkhel, B.C., Thakur, M.S., Parmar, S.N.S., Tomar, S.S. and Das, G. (2015).. Genetic polymorphism at exon-2 MHC B-LβII family gene by PCR-SSP in Kadaknath chicken. ***Journal of Interacademicia (*Accepted*).***
  NAAS Rating: ***2.34***

• Research Articles sent for Publication:

- Thakur, M.S., Parmar, S.N.S., Amit Kumar, Tomar, S.S., Sarkhel, B.C. and Das. G. (2016). Response of Cari Vishal and Cobb broiler chicks to experimental infection with *Eimeria tenella.* ***Journal of Veterinary Parasitology*** (***Under Review***).

  Article Submission Acknowledgement: Ref: DE/IJOR/SUB/jvp/24061522555

  Submission Date: 24 Jun, 2015

  NAAS Rating: ***4.72***

- Thakur, M.S., Parmar, S.N.S., Amit Kumar and Tomar, S.S. (2016). Molecular characterization of B-Lβ II family alleles in Caribro-Vishal chicken by PCR-SSP. ***The Indian Journal of Veterinary Sciences and Biotechnology*** (***Under Review***).

  Article Submission Acknowledgement: Ref: DE/IJOR/SUB/ijfv/0911152819

  Submission Date: 09 Nov, 2015

  NAAS Rating: ***3.2***

• Abstract published in Seminar/Conference/Symposia:

- Kumar, Amit, Thakur, M.S., Tomar S.S. and Parmar, S.N.S. (). Molecular characterization of B-Lβ II family alleles in Hubbard chickens. *In:* International symposium on sustainable management of animal genetics resources for livelihood security in developing countries & XII Annual convention of Society for Conservation of Domestic Animal Biodiversity (SOCDAB) held on Feb. 13-14, 2015, Madras Veterinary College Chennai.

- Thakur, M.S., Amit Kumar, Parmar, S.N.S. and Tomar, S.S. (2015). Characterization of genetic polymorphism of B-Lβ II family alleles in Ven-Cobb chickens. *In:* International symposium on sustainable management of animal genetics resources for livelihood security in developing countries & XII Annual convention of society for conservation of domestic animal biodiversity (SOCDAB) held on Feb. 13-14, 2015, Madras Veterinary College Chennai.

- Thakur, M.S., Parmar, S.N.S., Tomar, S.S. and Amit Kumar (2015). Differential expression of cytokine genes in Cobb, kadaknath and Caribro-91 chickens against coccidial challenge. *In:* National seminar on translational research in biotechnology for improving livestock health & production held on Oct. 7-8, 2015, Department of Veterinary Microbiology & Biotechnology, College of Veterinary and Animal Science, Rajasthan University of Veterinary and Animal Science, Bikaner.

- Thakur, M.S., Parmar, S.N.S., Sarkhel, B.C., Tomar, S.S. and Amit Kumar (2015). Relationship of MHC genes polymorphism with coccidial resistance in chicken. *In:* XXXII Annual Conference of Indian Poultry Science Association (IPSACON 2015) and National Symposium on "Green and clean poultry production" held on Nov. 19-21, 2015, CASM, Thiruvazhamkunnu, Palakkad (Kerala).

- Amit Kumar Jha, M. S. Thakur, S.N.S. Parmar, S.S. Tomar and B.C. Sarkhel (2016). Genetic differences in susceptibility and differential expression of cytokine genes in relation to coccidial challenges in Naked Neck, Hubbard and Dual Coloured type Chicken. *In:* National symposium on policy planning for livelihood security through domestic animal biodiversity & XIV Annual convention of Society for conservation of domestic animal biodiversity held on Feb. 11-12, 2016, R.S. Pura, Jammu, J& K.

- M. S. Thakur, S.N.S. Parmar, S.S. Tomar and Amit Kumar Jha (2016). Differential expression of cytokine genes in Cobb, Kadaknath and Caribro-91 chickens against coccidial challenge. *In:* National symposium on policy planning for livelihood security through domestic animal biodiversity & XIV Annual convention of Society for conservation of domestic animal biodiversity held on Feb. 11-12, 2016, R.S. Pura, Jammu, J& K.

- **Project Publicity in NEWS:**
  - ✓ The projects funded by M.P. Biotechnology have been covered by Dainik Bhasker in City Bhaskar page, Jabalpur on 19 April, 2016.

- **PhD research work under the project:**

One of the important achievement of this project is that the birds of different lines/breed maintained under the project were used as **valuable resource material for two Ph.D. research work**.

1. PhD thesis title: **Major histocompatibility complex gene polymorphism and expression of cytokine genes in relation to coccidial resistance in poultry***

   Breed targeted*: Kadaknath, Cobb broiler and Caribro-91

   Name of student: Dr. M.S. Thakur *(Degree awarded in year 2015)*

   Major Advisor: Dr. S.N.S. Parmar

   Discipline: Animal Genetics and Breeding

2. PhD thesis title: **Polymorphism of major histocompatibility complex gene and expression profile of cytokine genes in relation to coccidial resistance in poultry#**

   Breed targeted#: Hubbard, Dual Colour type and Naked Neck

   Name of student: Dr. Amit Kumar Jha *(Degree awarded in year 2015)*

   Major Advisor: Dr. B.C. Sarkhel

2. Details of patent filed-if any: Nil

As the work carried out under this project was to find out the genetic resistance or susceptibility in relation to coccidial challenge in different breeds/lines of chicken. The screening genetic resistance or susceptibility of birds was carried out using the most important coccidial resistance parameters as per the standard techniques. So, no patent is filled.

3. A brief of technology developed-if any, or it's possibilities:

We have standardized the level of lesion score, oocyst index value, OPG count range for identification of the most resistant or susceptible genetic group/s among six breeds/strains (i.e., **Kadaknath, Jabalpur dual coloured, Cobb, Hubbard, Naked Neck and Caribro-91**). Policy could be formulated to include the resistant breed/s in future poultry breeding plans in Madhya Pradesh. There is a possibility to ccharacterized the dynamics of chicken cytokine responses associated with *E. tenella* infection. This will provide a rational basis for use of cytokines and chemokines as therapeutic agents against coccidiosis and this will lessen the drug based control measures. A non-drug mediated control strategy using genomics, molecular biology and immunology for other poultry diseases thus can be initiated.

4. A brief para about "application and use of research outcomes-the Technology Aspect"

The present study was conducted to find out the MHC B-Lβ family (class II) alleles polymorphism in chicken using PCR-SSP and relationship with coccidial resistance in chicken. A total of 360 unsexed day old broiler chicks, sixty each of Kadaknath, Caribro-91(Cari Vishal), Jabalpur Dual Coloured, Hubbard, Naked Neck birds and Cobb broiler chicken, were included in the present study. Birds were challenged by gavaging 10,000 sporulated oocyst on 21st days of age. The OPG count, lesion score, Oocyst index and weekly body weight (0 to 8 weeks of age) were recorded of each genetic group. Blood samples were collected from each of the birds and genomic DNA was isolated. A 235bp, exon-2 region of chicken MHC BL-β II family gene was amplified using a set of five primers *i.e.,* B2 (222bp), B13 (141bp), B15 (222bp), B19 (213bp) and B21 (213bp). The number of genotypes found in Cobb, Kadaknath, Caribro-91, Naked Neck, Hubbard and Jabalpur Dual Coloured were 9, 8, 10, 10, 8 and 10, respectively. The genotype $B_{13}B_{21}$ was absent in all the genetic groups. The birds with genotype B_2B_{21} and $B_{13}B_{19}$ gained higher body weight and also least affected by coccidial infection in comparison to other genotypes among all the genetic groups. The coccidial infection significantly affected the body mass gain and feed conversion ratio of the birds. The Mortality amongst the different genetic groups ranged from 0 to 15 %. **The lowest mean for lesion score, oocyst index and OPG were found in Kadaknath as compared to Jabalpur dual**

coloured, Cobb, Hubbard, Naked Neck and Caribro-91 genetic groups. The Cobb genetic group was most susceptible whereas Kadaknath genetic group was most resistant to coccidial infection. The findings of project like OPG count, lesion score, Oocyst index etc. can be used for assessment of genetic resistance of different lines or breeds of poultry.

5. A brief para about "application and use of research outcomes-for the state of Madhya Pradesh"

The project research work provides a clue to the resistance capacity of the local Kadaknath birds as compared to the multicross varieties, suggesting for the incorporation of local germplasm into the new varieties. These results will definitely contribute to the further understanding of the genetic and immunological basis of resistance to coccidial infection in poultry. These findings suggest that there may be a room for some latitude in utilization of Kadaknath breed of chicken for the development of coccidial resistance commercial lines/strains of birds in future, thereby increasing the production efficiency of farmers flock of Madhya Pradesh.

6. Further research potential and support required from the council, if any.

In future, I am interested to continue this work under new project taking greater sample size with larger regions of MHC genes for ascertaining and utilizing the genetic variations against coccidial resistance. Other breeds of chicken may be studied to explore the association of genotypes at this region of MHC gene with some other coccidial resistance parameters namely, plasma carotenoids and plasma NO_2^- + NO_3^- can also be taken for association study of MHC gene along with differential expression of cytokine genes in response to coccidial challenges with different dosages of sporulated oocysts of *Eimeria* species. The relative expression of the cytokine genes could play a crucial role by driving higher immune responses in susceptible birds to coccidial infection. MHC gene along with growth hormone gene can also be taken for association study of body weight in relation to coccidial resistance in chickens. In future, we are planning to submit new R&D project on above aspect aiming to explore all the possibilities genetic basis of disease resistance in poultry.