[Frontiers In Bioscience, Elite, 10, 506-519, June 1, 2018]

MHC-B haplotypes impact susceptibility and resistance to RSV-A infection

Vishwa M. Khare[1], Vishesh K. Saxena[2], Alka Tomar[3], K.P. Singh[4], Kunwar B. Singh[5], Amit K. Tiwari[6]

[1]Cell and Developmental Biology, School of Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA, [2]Disease Genetics and Biotechnology Lab, CARI, Izatnagar, UP-243 122, India, [3]Tumor Immunology Lab, IVRI, Izatnagar, UP-243 122, India, [4]CADRAD, IVRI, Izatnagar, UP-243 122, India, [5]Animal Science Department, Rohilkhand University, Bareilly (UP), India, [6]Department of Pharmacology and Experimental Therapeutics, The University of Toledo, Toledo, OH 43614, USA

TABLE OF CONTENTS

1. ABSTRACT

We investigated the impact of haplotype of major histocompatibility complex (MHC)-B on the outcome of infection of Synthetic Dam Line (SDL) broiler strain with Rous Sarcoma Virus (RSV). Genomic analysis of MHC-B haplotypes, revealed a total of 12 known standard haplotypes that constituted to twenty-five different genotypes and one new haplotype of 217 bp size, designated B^X. The inoculation of RSV-A in SDL chicks resulted in the development of tumors of progressive or regressive phenotypes with varying tumor profile index (TPI). Haplotypes B^2, B^{21} and B^{22} had low TPI scores (1 or 2) with less mortality and were resistant to RSV-A tumor. The haplotypes B^{13}, $B^{13.1.}$, B^{15}, $B^{15.1.}$ and $B^{15.2.}$ had significantly higher TPI scores (5 or 6), indicating a susceptibility to RSV-A. The genotype, B^x /B^x, had a mean TPI score of 3.67 ± 1.33, which was closer to the resistant haplotype. Sequence analysis of the new haplotype (B^X) revealed 99.5% similarity with B2 haplotype. Metastases was observed in 44% of chicks and comprised of mixed fibrosarcoma and myxosarcoma.

2. INTRODUCTION

Avian leucosis or lymphoid leucosis is the most common form of neoplastic diseases observed in chicken and other avian species (1). Most of the neoplastic diseases have a viral etiology and are caused by avian retroviruses of genus *Alpharetrovirus* of *Retroviridae* family (2). These diseases cause significant economic losses in commercial layers and breeder flocks due to tumor mortality and other reproductive problems such as delayed sexual maturity, low fertility and hatchability and drop in egg production (3). Moreover, avian leucosis virus (ALV) is present in commercial chickens and eggs, thus exposing human beings on a consistent basis. Till date, since no commercial vaccines are available for control of ALV infection, improving the genetic attribute to disease resistance is the best approach for sustainable control of ALV infectious diseases in poultry.

Genetic resistance to diseases is a multigenic trait controlled primarily by the immune system and their interactions with physiological and environmental factors (4). In order to develop genetically resistant breeds, it is essential to recognize the immunological factors that are responsible for differences in natural resistance (5). The diversity of the major histocompatibility complex (MHC), T cell receptors (TcR) and immunoglobulins (B cell receptors) constitute the primary immunological basis for variations in disease resistance (4). In chickens, the association between MHC polymorphisms and susceptibility to infectious pathogens is well established (6, 7). Indeed, MHC protein polymorphisms have been shown to have a significant positive correlation with resistance or susceptibility to numerous autoimmune, viral, bacterial, and parasitic diseases, including avian leucosis (8) and Rous sarcoma virus (RSV) tumors (9, 10).

Disease resistance studies in Rous sarcoma virus have allowed new findings on related mechanisms and the genes involved (10). Analysis of inbred lines, their crosses, congenic lines, and noninbred populations have revealed the anti-RSV response of many B complex haplotypes (10). Particular MHC genotypes determine RSV tumor regression or progression and based upon degree of tumor growth, a tumor profile index (TPI) was assigned (11).

MHC haplotypes are being determined from genomic DNA by PCR using a polymorphic microsatellite marker LEI0258 and MCW0371 (12), which is located within the MHC region on chromosome 16 (13) between BG and BF region or sequence-based typing (SBT) of the MHC-B locus class I α chain 2 (BF2) allele (14). Recently, Suzuki (15) investigated the relationship between MHC-B haplotypes and response to RSV-J in an F2 commercial chicken family by microsatellite marker LEI0258, BF2 SBT and an additional 35 SNP to discriminate more precisely between regression and progression phenotypes.

The variation in the resistance/ susceptibility of high yielding synthetic dam line (SDL) a broiler strain of India to RSV-A has not been studied so far. Therefore, this study was designed to explore the prevailing MHC haplotypes/ genotypes in the Synthetic Dam Line (SDL) broiler strain and their association with resistance and susceptibility to RSV-A. The MHC haplotypes of the SDL chicks were determined using genomic DNA as a template before challenging the chicks with RSV-A. Genotyping was accomplished using the PCR microsatellite markers LEI0258 and MCW0371 located within the MHC region.

The information on MHC haplotypes of the Indian native SDL broiler to Rous sarcoma virus will help in identifying chickens with susceptibility and resistance phenotypes and in their conservation through targeted strategies for control of tumor outcome.

3. MATERIALS AND METHODS

3.1. Virus

Bryan Standard strain of Rous sarcoma virus (Rous associated virus-1) (BS-RSV (RAV-1)), henceforth to be referred as RSV-A, was used for the present study.

Freeze dried ampoules of RSV-A (Rous sarcoma virus) obtained from Tumor Immunology Lab,

Table 1. Primers For MHC-B Haplotyping

S. No.	Primers	Sequences	Accession No.	Allele size range
1	LEI0258	(F) CAC GCA GCA GAA CTT GGT AAGG (R) AGC TGT GCT CAG TCC TCA GTGC	AL023516	182-552 bp
2	MCW0371	(F) CTG CTC CGA GCT GTA ATC CTG (R) TTT CAT GGC ATC CTA AGA TG	AL023516	200-209 bp

The PCR products of individual DNA samples were resolved on a 4% MetaPhor agarose gel.

IVRI, Izatnagar were used for the fresh preparation of RSV. Each freeze dried ampoule was reconstituted in 0.5 ml of Normal saline solution (NSS). 9 to 11 day old embryonated chick eggs procured from Experimental Broiler Farm, CARI, Izatnagar were utilized to determine titer of Rous Sarcoma Virus (RSV) by chorioallantoic membrane (CAM) assay as per the method of Groupe *et al*. (16). Infectivity titer of the virus was found to be $1x10^3$ pock forming units (p.f.u.) / ml. The virus was handled under biosafety level 2 facilities.

3.2. Chicks and management practices

The protocols involving the care and use of animals for these experiments were in accordance with the with the rules of the Animal Ethics Monitoring Committee of the Institute, Government of India.

One day old chicks, belonging to white-plumaged Synthetic Broiler Dam Line (SDL), were obtained from the Experimental Broiler Farm, Central Avian Research Institute (CARI), Izatnagar Uttar Pradesh (UP), India. The chicks were shifted to challenge sheds at the Indian Veterinary Research Institute (IVRI), Izatnagar, UP, India, where they were infected with Rous sarcoma virus (RSV-A). The birds were housed in battery brooders and were maintained under standard management and nutrition. The chicks were brooded under controlled warm conditions and were fed *ad lib* starter ration (upto 3 week) and finisher ration thereafter (upto 5 week). They had free access to feed and water. Individual chicks were wing banded for identification. A group of uninfected control chicks was simultaneously maintained in the same facility.

3.3. MHC haplotyping

We determined the MHC haplotypes of 80 SDL chicks using PCR-based genotyping of microsatellite sequences within the MHC region, using the method of Fulton *et al*. (12). We used genomic DNA from pre-challenged chicks with RSV-A, as a template and LEI0258 as a microsatellite marker. MHC haplotypes that could not be distinguished using the LEI0258 microsatellite alone were distinguished by the MCW0371 marker. The details of primer sequences used for haplotyping are given in Table 1.

3.4. Tumor induction

One week old SDL broiler chicks were subcutaneously infected with Rous Sarcoma Virus (2000 p.f.u/0.2 mL of RSV-A suspension per chick) in the left wing-web and reared in the Challenge Sheds, IVRI, Izatnagar under contained facilities. The droppings, dead and necropsied birds, and other discards were disposed through incineration. Chicks were observed regularly for the appearance of tumors at the primary site, as well as in other organs. Uninfected control birds were maintained separately, observed daily, and sacrificed by cervical dislocation at the end of the experiment.

3.5. Categorization of chicks

The growth pattern of the primary tumors induced by Rous sarcoma virus was observed every day, and chicks were categorized according to the magnitude of the growth of the tumor. The scoring was done to assign a tumor profile index (TPI) (17) using the following: TPI-1= no palpable tumor; TPI-2= complete regression by 42 or 56 days; TPI-3= complete regression by 70 days, a decreasing slope, or complete regression by 56 days followed by recurrence; TPI-4= tumor covering > half the wing web area, but < entire wing web; TPI-5= tumor filling the entire wing web; or TPI-6 = massive tumor extending beyond the wing web and metastasizing to other organs. Chicks with a TPI score of 1 were deemed non-responders, 2 or 3 were regressors and 4, 5 and 6 were progressors.

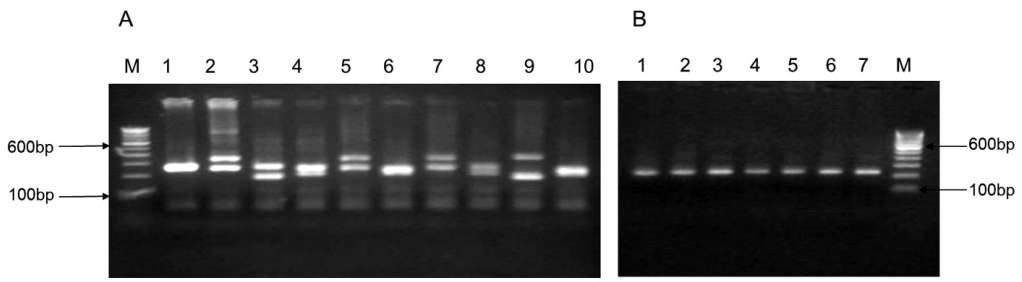
3.6. Gross pathological examinations

The growth pattern of the primary tumors at the wing web that was induced by Rous sarcoma virus was assessed by measuring the volume of primary tumor with the help of vernier calipers every day. Dead chicks were necropsied for gross pathological findings in different organs of the body and the site of gross lesions were recorded.

3.7. Histopathological examinations

3.7.1. Collection of samples for histopathology

The experimental chicks were sacrificed by cervical dislocation and tissue samples *viz.,* primary



Lane M : 100 bp ladder
Lane 1 : B2/B2 (261 bp)
Lane 2 : B15.2/B21 (249 and 357 bp)
Lane 3 : B13/B15 (205 and 261 bp)
Lane 4 : Bx/B15.2 (217 and 249 bp)
Lane 5 : B15.2/B21 (249 and 357 bp)
Lane 6 : Bx/Bx (217 bp)
Lane 7 : B15.2/B21 (249 and 357 bp)
Lane 8 : Bx/B15.2 (217 and 249 bp)
Lane 9 : B13/B21 (205 and 357 bp)
Lane 10 : B15/B15 (261 bp)

Lane 1 : B13 (202 bp)
Lane 2 : B15 (203 bp)
Lane 3 : B15 (203 bp)
Lane 4 : B2 (206 bp)
Lane 5 : B2 (206 bp)
Lane 6 : B15 (203 bp)
Lane 7 : B15 (203 bp)
Lane M : 100 bp ladder

Figure 1. Amplification of MHC-B Haplotypes using (A). LEI0258 microsatellite (B). MCW0371 microsatellite.

tumor tissue, lung, liver, spleen, heart, kidney, neck and leg muscle were collected from progressor birds and fixed in 10% neutral buffered formalin solution for histopathological studies.

3.7.2. Histopathological procedure

Tissue pieces were microsectioned into 2-3 mm and washed overnight with running water. The samples were then dehydrated with ascending grades of ethanol starting from 70% ethanol, to 80%, 90% and 95%, absolute ethanol for 1 hour at each grade. Following dehydration, the tissues were cleared in three changes of xylene for 1 hour for each change. The tissue pieces were embedded in paraffin and sections were cut at 4 microns. These microsections were fixed on slides and the sections were stained with hematoxylin and eosin (H&E).

3.7.3. Hematoxylin and Eosin (H & E) staining procedure

Sections were deparafinized in three changes of Xylene for 10 min. for each change. The sections were rehydrated in descending grades of ethanol (90%, 80%, 70%) and finally brought to water. The sections were stained with H & E stains and finally mounted in DPX (Distyrene Plasticizer Xylene). Sections were examined under compound microscope.

3.8. Statistical analysis

3.8.1. Effects of MHC-genotypes on tumor profile index (TPI)

The fixed effect of MHC genotypes on TPI was evaluated using SPSS version 16.0. The MHC genotypes were coded and analyzed using following model:

$$Y_{ij} = \mu + G_i + e_{ij}$$

Where,

Y_{ij}=TPI under i^{th} genotype

μ =Overall mean

G_i=Effect of i^{th} MHC-genotype

e_{ij}=Random error distributed with mean 0 and variance s^2

4. RESULTS

4.1. Determination of various MHC haplotypes and genotypes

Numerous studies have been conducted globally to identify different MHC haplotypes present in different chicken breeds (12, 18, 19). However, there are scanty reports on Indian chicken germplasm and this investigation is the first report on indigenously developed broiler parent stock in India. Therefore, the present investigation was conducted on SDL chicks using PCR- based genotyping of the genomic DNA by LEI0258 and MCW0371 microsatellite markers (12), which are located within MHC region on chromosome 16 (13) between BG and BF region. The band sizes of different haplotypes were detected using a 4% MetaPhor agarose gel and categorized into different B haplotypes based on their LEI0258 polymorphic microsatellite marker sizes (Figure 1A). Allele size variations for LEI0258 ranged from 182 bp to 552 bp and ranged from 200 bp to 209 bp for MCW0371. The MHC haplotypes that shared a common LEI0258

Table 2. Different MHC haplotypes and their sizes with LEI0258 and MCW0371 microsatellite marker

S.No.	Haplotype	LEI0258 size (bp)	MCW0371 size (bp)
1	B2	261	206
2	B8	405	206
3	B12.3	513	205
4	B13	205	202
5	B13.1	381	206
6	B15	261	203
7	B15.1	193	200
8	B15.2	249	206
9	B21	357	205
10	B22	249	206
11	B72	307	208
12	BX	217	206

Figure 2. Sequence of (A). BX, New B-haplotype (217 bp); (B). Alignment of 217 bp pair sequences with B2 haplotype.

allele size were distinguished by the MCW0371 marker (Figure 1B). It was observed that the B^2 and B^{15} haplotypes had an identical allele size of 261bp for LEI0258. However, their MCW0371 allele differed (B^2=206, B^{15}=203) such that these two haplotypes could be distinguished with this marker.

MHC-B haplotypes results revealed the following twelve haplotypes: B^2, B^8, $B^{12.3}$, B^{13}, $B^{13.1}$, B^{15}, $B^{15.1}$, $B^{15.2}$, B^{21}, B^{22} , B^{72}, and B^x, a newly identified haplotype that was 217 bp in length (Table 2).

Further confirmation of identified B-haplotypes was done by cloning, sequencing and sequence alignment. A comparison of individual B-haplotypes sequences, using a BLAST algorithm, indicated that all the sequences belonged to *Gallus gallus* chromosome 16 genomic contig. The sequence of each haplotype was aligned with the reported sequence of each haplotype. The new sequence of 217 bp size (Figure 2A) showed high resemblance (99.5%) with the B2 haplotype (261), but had a deletion of 24 bp (Figure 2B). Further confirmation is therefore required to find out whether this is a new haplotype or a variant of the B2 haplotype.

The above 12 haplotypes were present in different combinations, constituting 25 different genotypes. The genotypes identified were: B^2/ B^2, B^2/ B^{13}, B^2/ $B^{15.1}$, B^8/ $B^{12.3}$, B^8/ B^{13}, B^{13}/ B^{13}, B^{13}/ $B^{13.1}$, B^{13}/ B^{15}, B^{13}/ $B^{15.2}$, B^{13}/ B^{21}, B^{13}/ B^{22}, B^{13}/ B^x, $B^{13.1}$/ B^{22}, B^{15}/ B^{15}, B^{15}/ $B^{15.1}$, B^{15}/ $B^{15.2}$, B^{15}/ B^{72}, $B^{15.1}$/ $B^{15.2}$, $B^{15.1}$/ B^{21}, $B^{15.1}$/ B^{22}, $B^{15.2}$/ $B^{15.2}$, $B^{15.2}$/ B^{21}, $B^{15.2}$/ B^x, B^{21}/ B^x and B^x/ B^x. Also, it was observed that most of the genotypes were present in the heterozygous condition and a few were in the homozygous condition. Only four genotypes viz. B^2/B^2, B^{13}/B^{13}, B^{15} /B^{15} and B^x/B^x were present in the homozygous condition. The occurrence

Table 3. Frequency and Mean tumor profile index (TPI) scores of different MHC Genotypes in SDL chicken population

S. No.	Genotype	Frequency	TPI (Mean ± SE)
1	B2/ B2	0.025	1.00 ± 0.00
2	B2/B13	0.013	2.01 ± 0.11
3	B2/B15.1	0.025	2.00 ± 0.00
4	B8/ B12.3	0.013	4.30 ± 0.45
5	B8/B13	0.013	4.83 ± 0.34
6	B13/ B13	0.063	5.50 ± 0.29
7	B13/ B13.1	0.025	6.00 ± 0.00
8	B13/ B15	0.038	5.00 ± 0.58
9	B13/ B15.2	0.163	4.92 ± 0.08
10	B13/ B21	0.025	2.00 ± 0.00
11	B13/ B22	0.113	2.11 ± 0.11
12	B13/ Bx	0.038	4.33 ± 0.67
13	B13.1/B22	0.013	2.14 ± 0.13
14	B15/ B15	0.038	6.00 ± 0.11
15	B15/ B15.1	0.013	5.45 ± 0.18
16	B15/ B15.2	0.038	5.33 ± 0.33
17	B15/ 72	0.013	5.77 ± 0.25
18	B15.1/ B15.2	0.05	4.50 ± 0.50
19	B15.1/ B21	0.05	5.25 ± 0.25
20	B15.1/ B22	0.025	2.00 ± 0.00
21	B15.2/ B15.2	0.025	5.00 ± 0.00
22	B15.2/ B21	0.05	4.75 ± 0.25
23	B15.2/Bx	0.075	5.67 ± 0.21
24	B21/Bx	0.025	2.00 ± 0.00
25	Bx/Bx	0.038	3.67 ± 1.33

of a higher proportion of heterozygotes might be due to selective advantage to heterozygotes for survival as well as the line has never bred as per MHC haplotypes (Table 3).

4.2. Frequency of different genotypes

The genotype frequencies of the SDL chicks ranged from 0.013 to 0.163. Genotype B^{13}/ $B^{15.2}$ was present at highest frequency (0.163), followed by B^{13}/ B^{22} (0.113), $B^{15.2}/B^{x}$ (0.075) and B^{13}/ B^{13} (0.063). Genotypes $B^{15.1}$/ $B^{15.2}$, $B^{15.1}$/ B^{21}, $B^{15.2}$/ B^{21} were at same frequency (0.050). Genotypes B^{2}/B^{13}, B^{8}/ $B^{12.3}$, B^{8}/B^{13}, $B^{13.1}/B^{22}$, B^{15}/ $B^{15.1}$, B^{15}/ B^{72} were present at a very low frequency 0.013 (Table 3).

4.3. Genetic manifestation of different B genotypes

Chicks of the genotype B^{2}/B^{2} did not develop any tumors during the entire period of the experiment; therefore, they were categorized as *non-responders.* The mean TPI score of 1.00±0.00 was assigned to this genotype, indicating that this genotype conferred resistance, resulting in the complete absence of tumors in these chicks. The tumors with the $B^{2}/B^{15.1.}$, B^{13}/B^{21}, B^{13}/B^{22}, $B^{15.1}/B^{22}$, and B^{21}/B^{x} genotypes chicks regressed and had a very low mean TPI (2.00±0.00 to 2.11±0.11). Thus, they were categorized as *regressor*s. Chicks with the genotypes B^{13}/B^{15}, $B^{13}/B^{15.2}$, B^{13}/B^{x}, $B^{15}/B^{15.1}$, $B^{15}/B^{15.2}$, $B^{15.1}/B^{15.2}$, $B^{15.1}/B^{21}$, $B^{15.2}/B^{15.2}$, $B^{15.2}/B^{21}$, and $B^{15.2}/B^{x}$ had progressive tumors, with mean TPI scores from 4.33 ±0.67 to 5.67±0.21. They were therefore categorized as *progressors.* Genotypes B^{13}/B^{13}, $B^{13}/B^{13.1}$, and B^{15}/B^{15} had the highest TPI scores (5.50±0.29 to 6.00±0.11). These tumors extended beyond the wing web and metastasized to other organs.

Genotype B^{x}/B^{x} had mean TPI score of 3.67±1.33 which indicated resistance of this haplotype towards Rous sarcoma virus. It was observed that if this haplotype was present in homozygous condition it made the bird resistant towards Rous sarcoma and if present in heterozygous condition in combination with resistant haplotype it became resistant genotype as indicated in genotype B^{21}/B^{x} with TPI 2.00±0.00 and if present with susceptible haplotype as in genotype $B^{15.2}/B^{x}$ the TPI score was increased to 5.67±0.21 indicating

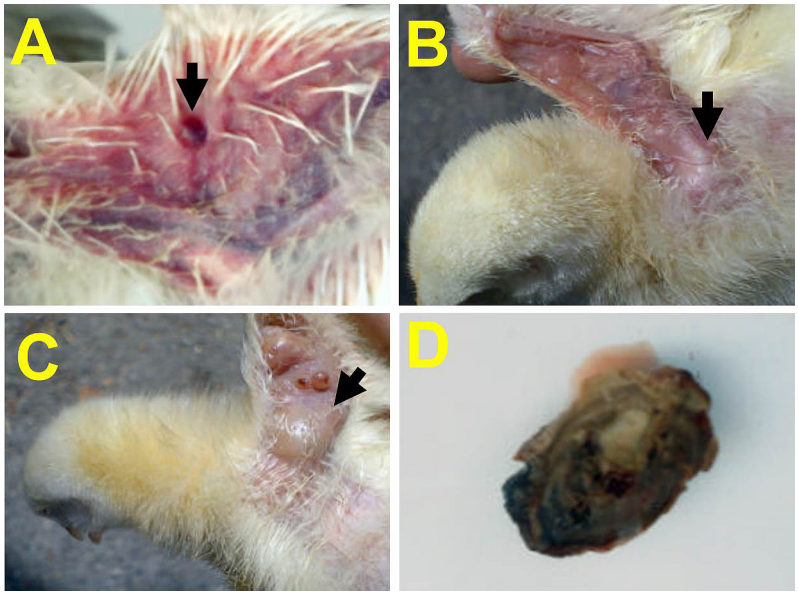


Figure 3. Primary solid tumor (A). as a single nodule on wing web (B). coalesced (C). covering whole wing web (D). transverse section.

its susceptibility towards Rous sarcoma virus. Thus, the haplotype Bx might be resistant recessive (Table 3).

4.4. Gross pathology

The inoculation of SDL chicks with RSV-A resulted in the appearance of a primary tumor at the site of inoculation between 2–10 days post infection. The tumor appeared as either a single pin head sized nodule or as multiple beads that coalesced later on to form one tumor mass (Figure 3A & 3B). The tumor grew rapidly and eventually involved the whole wing web, which restricted the movement of the bird (Figure 3C). Tumors varied in consistency from a soft palpable to hard, solid nodular mass (Figure 3D). Soft and palpable tumors contained reddish – brown, slimy tenacious material with foul smelling fluid. The solid tumor mass had dirty, white - colored cheesy material and in some tumor masses, concentric rings were observed upon cross-section.

In addition to the primary tumor, some chicks developed metastatic tumors in different organs of the body and on the skin (Figure 4A). Post-mortem reports revealed metastasis in the mandibular region, covering the jaw, tongue (Figure 4B), neck and thymus (Figure 4C), the area between the rib bones and chest muscle (Figure 4D) and leg muscle (Figure 4E). Pearl - like white nodules were seen on the surface of the lung, liver, spleen, heart and kidney (Figure 4F - 4J). The liver and spleen had multiple, white colored pearl - like lesions that were friable and soft and were 1mm to 5mm in diameter. The lung, heart and kidney had small, pin-point white foci. The chest, neck and leg muscles were thickened due to the proliferation of cells. The proventriculus, gizzard and caecum were also affected and showed micro-foci on their surfaces.

The percentage of chicks showing metastasis in different organs are shown in Table 4. In the present study, the incidence of metastasis was mostly observed in the lung, liver and heart (24%), followed by the spleen (10%) and kidneys (8%). The least affected organs were the proventriculus, gizzard, caecum, intestine and serosa (2%). Splenomegaly and hepatomegaly was also observed but the bursa was not affected in any of the chicks.

4.5. Histopathology

Histopathological analysis typically revealed two types of tumors which were fibrosarcoma and myxosarcoma. Fibrosarcoma consisted of immature fibroblasts, loosely arranged in irregular interwoven bundles, with a moderate amount of collagen. Myxosarcoma was characterized by stellate – like or fusiform - shaped tumor cells, with extended cytoplasm. The tumor cells were homogenous, with slightly basophilic mucinous contents. However, in few birds, myxofibrosarcoma was also observed.

4.5.1 Progressor birds

4.5.1.1. Primary tumor

At the site of inoculation, the tumors appeared as fibrosarcoma. In most cases, a typical fibrosarcoma had degeneration of tissues in the center and pronounced infiltration of mesenchymal cells at the periphery. The cancer cells were spindle - shaped and multidirectional, with no set pattern. At some locations, the cancer cells were compact and at other locations, they appeared myxomatous. The cancer invaded muscle tissue, causing atrophy. The

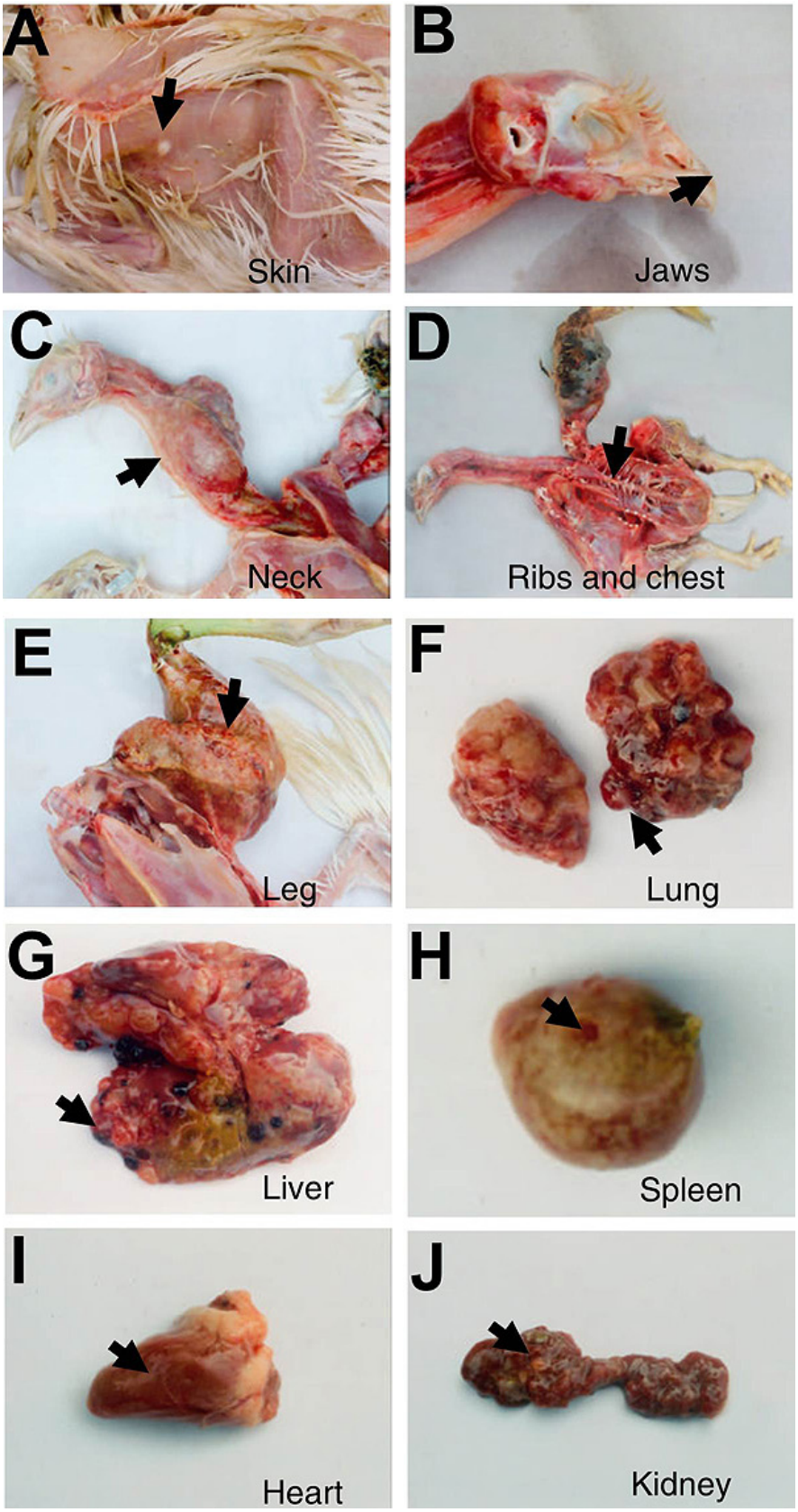


Figure 4. Representative tumor lesions metastasized to (A). Skin (B). Jaws (C). Neck (D). Ribs and chest (E). Leg (F). Lung (G). Liver (H). Spleen (I). Heart (J). Kidney.

Table 4. Percentage of chicks showing metastasis in different organs

S. No.	Organ	% of Chicks
1	Lung	24
2	Liver	24
3	Heart	24
4	Spleen	10
5	Chest	10
6	Kidney	8
7	Leg	8
8	Neck	6
9	Skin	6
10	Jaw & Tongue	2
11	Proventriculus	2
12	Gizzard	2
13	Caecum	2
14	Bursa	0
15	Splenomegaly	10
16	Hepatomegaly	12

primary tumors were highly vascularized, containing numerous and highly engorged blood vessels, as well as extensive hemorrhages. There was degeneration and necrosis of cells. The tumor stroma was infiltrated with neutrophils, mononuclear cells and plasma cells. There was proliferation of cells around hair follicles but the epidermis and skin were intact. Neoplastic cells were found in bundles (Figure 5).

4.5.1.2. Leg muscle

There was extensive proliferation of neoplastic cells, causing replacement and atrophy of muscle fibers. Fibrosarcoma caused extensive damage to the underlying muscle, such that the muscle fibers were ruptured and separated due to presence of erythrocyte-rich, edematous fluid between the fibers. There were different patterns of cancerous cells. They were found in compact, long spindle - shaped cells or loosely arranged with different orientations. There were whorl formations with no specific direction. The cancerous cells were highly vascularized, extensively myxomatous, with only remnants of muscle fibers and at a few places, the shape of the cells was oval in shape. The necrosis of the muscle cells, along with infiltration of inflammatory cells, was evident at certain places (Figure 6A).

4.5.1.3. Chest muscle

The chest muscles were completely replaced by neoplastic cells. Cells were oval - shaped and loosely arranged with myxomatous tissues in between the cells. Neoplastic cells were rarely of the fibrosarcomatous type. Groups of secretory cells were present in myxomatous tissues. The nature of the cells suggested the presence of an advanced stage of cancer (Figure 6B).

4.5.1.4. Lung

Histopathologically, both fibrosarcoma and myxosarcoma was observed, in combination with proliferation of lymphoid cells. The tumor cells had loosely arranged, elongated cutaneous cells with loss of orientation characterized by a whirling pattern. In some cases with myxosarcomatous tissues, cells were conglomerated to form a mass or clump of nuclei (Figure 6C).

4.5.1.5. Liver

Moderate to extensive tumor growths of varying size were present and replaced the normal hepatic parenchyma. Degenerative tissues, vacuolations, lymphocytic infiltration and hemorrhages were seen in the liver parenchyma. The perivascular area had mononuclear cell and fatty cell infiltration. There was proliferation of cutaneous cells, which were loosely arranged. Fibrosarcoma could be seen as well (Figure 6D).

4.5.1.6. Spleen

The spleen was infiltrated by loosely arranged lymphoid cells. Concentrated sarcomatous tissues were visible and the compact mass of cells that could be seen was fibrosarcoma (Figure 6E).

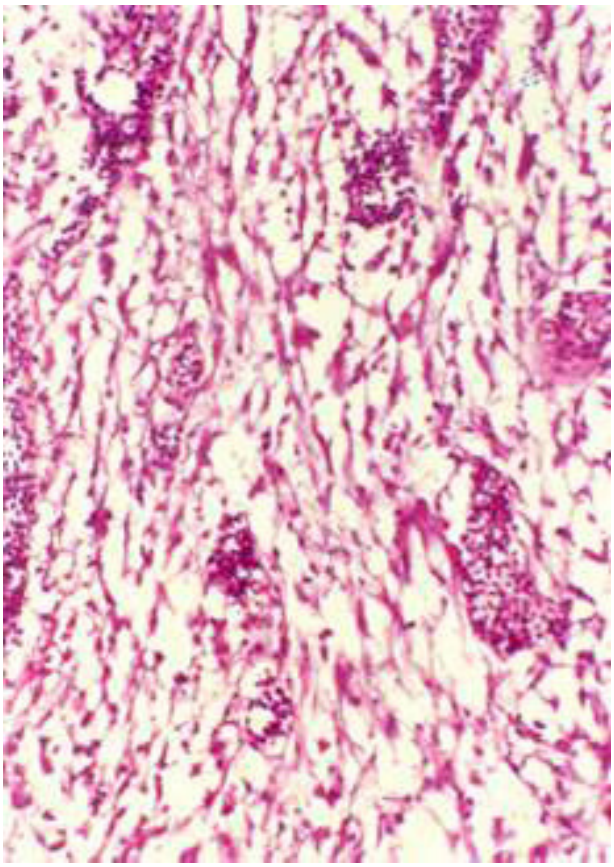


Figure 5. Primary tumor showing severe hemorrhages of RBCs of tumor tissue and presence of dense fibro-sarcoma (H&E; 25 x 6.3.).

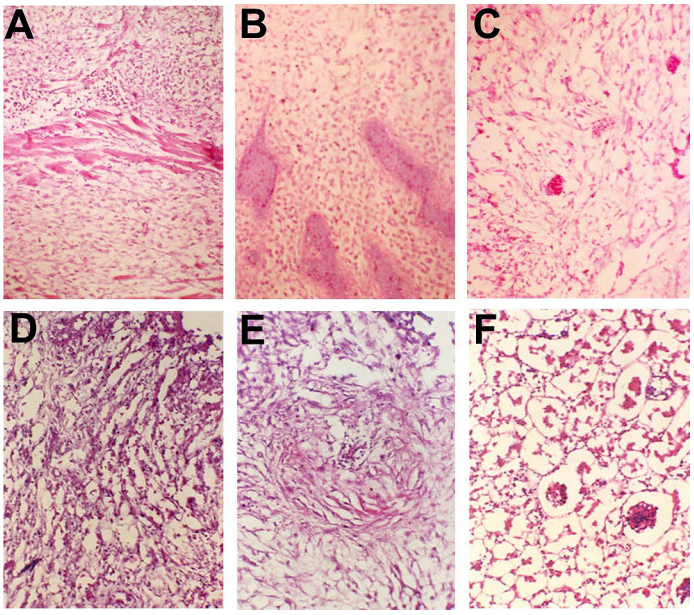


Figure 6. (A) Leg muscle showing proliferation of neoplastic cells causing atrophy of muscle fiber and presence of myxomatous cancerous cells that are loosely arranged with different orientation (H&E; 16 x 6.3.) (B). Presence of myxomatous cancerous cells with oval shape and loose arrangement in chest muscle (H&E; 25 x 6.3.) (C). Lung showing presence of both fibro-sarcoma and myxosarcoma, proliferation of lymphoid cells and elongated cutaneous cells that are loosely attached with loss of orientation in lungs (H&E; 25 x 6.3.) (D). Liver showing fibro sarcoma with degenerative changes, vacuolations lymphocytic infiltration and hemorrhages in parenchyma and proliferation of loosely arranged cutaneous cells (H&E; 25 x 6.3.) (E). Spleen showing compact cells of fibro-sarcoma and proliferation of loosely arranged lymphoid cells (H&E; 25 x 6.3.) (F). Kidney showing degeneration of kidney tubules and glomerulli and rupturing of RBCs with deposition of debris at the center (H&E; 25 x 6.3.).

4.5.1.7. Heart

The myocardial fibers were separated and showed degenerative changes due to the infiltrating tumor. A mixed type of cellular infiltration, with mononuclear cells and heterophils, were present (tissue slide not shown).

4.5.1.8. Kidney

There was degeneration of the kidney tubules and glomeruli. Ruptured RBC and debris were deposited in the center of glomeruli (Figure 6F).

4.5.2. Regressor birds

Lung, liver, spleen, heart, kidney and the site of inoculation were carefully examined histopathologically and there was no tumor formation was observed.

4.5.3. Non-Responder and uninfected control birds

Non-Responder and uninfected control birds also did not show any tumor formation in different the organs viz. Lung, liver, spleen, heart, kidney and the site of inoculation.

5. DISCUSSION

The present investigation was designed to determine the MHC-B haplotype status of the SDL broiler strain and the association of MHC haplotypes with cancer induction and progression. First, we determined the MHC haplotypes present in SDL chicks using LEI0258 microsatellite marker. We also used the MCW0371 marker when the haplotypes could not be differentiated by LEI0258 alone. Our study revealed twelve different haplotypes, constituting twenty-five different genotypes in combination. Apart from the standard haplotypes, a new, 217 bp haplotype was found and designated haplotype as B^x. The majority of the genotypes were heterozygous, with the frequency ranging from 0.013 to 0.163. The current study demonstrated a wide variety of MHC haplotype, which was in accordance with the findings of previous studies in domestic chickens (12, 18-20). Fulton *et al.* (12), identified 51 different genotypes with 26 distinctive alleles, using LEI0258 marker in chickens. Similarly, in Brazilian local chickens, using the LEI0258 microsatellite (19), 53 different genotypes, with 15 different alleles, were reported. Schou *et al.* (18) also used this marker to identify two different chicken breeds in Vietnam. In one breed, 19 different alleles were found and in another breed, 24 distinctive alleles were identified. Lwelamira *et al.* (20) reported the presence of 22 and 23 alleles of LEI0258 in the Tanzania *Kuchi* and *Medium* ecotypes, respectively. The number of alleles in these studies, including our work, were much higher. However, a lower number of alleles were reported for commercial breeds such as the Lohman Silver Line (3 alleles) and the Lohman Brown line (5 alleles) (18). The presence of a higher number of alleles in the SDL chickens suggested greater variability at their MHC locus.

To elucidate the genetic manifestation of the different B genotypes, the overall tumor growth pattern was observed and a tumor profile index (TPI) score was determined. A higher TPI indicated greater progression of tumor growth, and thus greater susceptibility to the Rous sarcoma virus. Conversely, a lower TPI indicated resistance towards the virus, as demonstrated by either no induction or regression of the tumor. Based on the TPI scores, it was noted that haplotypes B^2, B^{21}, and B^{22} were resistant, as these SDL chicks had a lower TPI score (1 or 2). Haplotypes B^{13}, $B^{13.1.}$, B^{15}, $B^{15.1.}$, and $B^{15.2.}$, however, were deemed more susceptible as their TPI scores were from 4 to 6. We also observed that chickens with the haplotypes B^2, B^{22}, and B^{21} showed reduced mortality and increased survival, further corroborating our hypothesis that these haplotypes confer resistance to RSV-A induction. Similar results were obtained by Collins *et al.* (11), who obtained TPI scores in two highly inbred lines homozygous for B^2 and B^5. They reported that the most resistant genotype, $B^2B^{2.}$ had a 5% mortality rate and a mean TPI of 2.94, whereas the most susceptible genotype, $B^5B^{5.}$ had a 93% mortality rate and a mean TPI of 4.93, and the heterozygotes had TPI values similar to the resistant genotype compared to those that were deemed susceptible to infection.

Furthermore in our study the haplotypes B^2 and B^{22} appeared to be resistant and dominant, as their presence in both homozygous and heterozygous conditions led to the reduction in TPI scores. The presence of these haplotypes in the homozygous condition (B^2/B^2) produced chicks that were non-responders. These chicks demonstrated no tumor development and their TPI scores were 1.00±0.00, indicating complete resistance against the Rous sarcoma virus. In the heterozygous condition, such as in genotype $B^2/B^{15.1}$, the presence of the B^2 allele reduced the TPI score (2.00), leading to tumor regression hence, indicating an incomplete dominance effect.

Various studies have reported that MHC complementation results in better outcomes compared to the homozygous condition. The susceptible homozygous genotypes B^{13}/B^{13}, $B^{13.1}/B^{13.1}$, and B^{15}/B^{15} had high TPI scores, ranging from 5.50±0.29 to 6.00±0.11, indicating their high susceptibility to RSV. Heterozygous genotypes had intermediate TPI scores between the susceptible and resistant homozygous genotypes. Senseney *et al.*, (21) also found MHC complementation between B^Q and B^{17} haplotypes in a Line UCD 001 (B^QB^Q) × UCD 003 ($B^{17}B^{17}$) cross. In addition, heterozygote animals had lower TPI scores

than the homozygote animals. Genotype B^{23}/B^{26} had a lower TPI compared with either the B^{23}/B^{23} or B^{26}/B^{26} homozygotes (22, 23) and the B^{22}/B^{26} genotype had a TPI lower than the B^{22}/B^{22} or B^{26}/B^{26} homozygotes (24). However, Taylor *et al.,* (25) suggested negative complementation between haplotypes B^{24} and B^{30}. Heterozygous $B^{24}B^{30}$ chickens had a higher TPI than B^{24}/B^{24} and B^{30}/B^{30} chickens but only the B^{24}/B^{30} and B^{30}/B^{30} types were significantly different.

We observed that the resistant haplotype B^{21} was dominant over B^{13} and B^{x}, but recessive to $B^{15.1}$ and $B^{15.2.}$. When haplotype B^{21} was present, along with B^{13} or B^{x}, the tumor regressed; however, when it was present with $B^{15.1.}$ or $B^{15.2.}$, the tumor progressed (TPI score of 5 to 6). Furthermore, it is worthwhile to emphasize that the newly identified B^{x} /B^{x} genotype had a mean TPI score of 3.67±1.33, which was closer to that of the resistant haplotype. Complementation of this haplotype, with a resistant haplotype, such as B^{21}, reduced the TPI score and conferred resistance. However, when B^{x} was combined with a susceptible haplotype, the TPI score was increased, indicating susceptibility to RSV. Similar results were reported by Taylor *et al.*, (25) and Lukacs *et al.,* (24), while studying the anti-tumor response of the B^{30} haplotype. Their results indicated that in the homozygous condition, B^{30}/B^{30} was more regressive; however, in the heterozygous state, it had a variable anti-tumor response. The combination with a progressive haplotype such as B^{24} resulted in a more progressive state, whereas coupling with a regressive haplotype such as B^{22} resulted in more regressive state. Thus, we concluded that in our study, haplotype B^{x} is likely to be resistant but recessive.

Based on our results, the order from dominant to recessive, was: B^{2} > B^{22} >B^{15} > $B^{15.1}$ >$B^{15.2}$ > B^{21}>B^{13} > B^{x} for RSV-A tumor resistance. We examined the gross pathology of the tumors and noted that primary tumors first appeared as a single pin - head sized nodule that grew and became extensive, covering the whole wing-web. Halpern *et al.,* (26) also found RSV - induced tumors highly enlarged, which reflected both tumor cell proliferation and viral replication generating new tumor cells. In the present study, the lung, liver and heart were the most affected organs, followed by the spleen and kidney and the least affected organs were proventriculus, gizzard, and intestine. Similar findings were reported by Collins *et al.* (27),where the incidence of metastatic tumors in the heart and pericardial sac was 27%, followed by the liver and pancreas (18%), gastrointestinal tract (15%) and the least affected organ was the kidney (2%).

Comparing the histopathological lesions in different organs of chicks, two types of sarcomas were encountered in progressor birds; however the regressor, non-responder and uninfected control did not show tumour in any of the organs studied. Mostly the tumors found in progressor birds were fibrosarcomas and to a lesser extent the myxosarcomas were found. Primary tumors had extensive fibrosarcomas, with compact to lose architecture in the proliferating neoplastic cells. Metastatic organs indicated a variable degree of infiltration of fibro and myxo sarcomatous tissues, causing atrophy of adjoining areas and replacing the original parenchyma, leaving only remnants of normal structure. Halpern *et al.,* (26) also found mostly fibrosarcomas, which consisted primarily of immature fibroblasts loosely arranged in irregular bundles. Histiocytic sarcomas as described by Arshad *et al* and Sastri *et al.* (28, 29) were not seen in this study which might be possibly due to a difference in the subgroup viral strain that was used.

6. CONCLUSION

The present study demonstrated a higher number of alleles in SDL chickens, suggesting greater variability at MHC locus. The role of MHC haplotypes in resistance/susceptibility was confirmed and the halpotypes B^{2}, B^{21} and B^{22} , exhibited resistance with dominance compared to the most susceptible haplotypes B^{13}, $B^{13.1}$, B^{15}, $B^{15.1}$ and $B^{15.2}$. Furthermore, in the homozygous condition, resistant haplotypes became more resistant to RSV, as indicated by the absence of tumor induction, while homozygosity in the susceptible haplotypes increased susceptibility. Genotypes in the heterozygous condition showed intermediate tumor growth between susceptible and resistant homozygous genotypes. Thus, we conclude that the heterozygous condition has better outcomes in tumor regression compared to the homozygous condition, except for B^{2}, B^{21} and B^{22}. Genetic complementation increased the response of heterozygotes compared to their component homozygotes. Although the exact mechanism of genetic complementation is not known, Brown *et al.*, (21) described the anti-tumor response as a complex reaction to various viral and tumor antigens. Heterozygotes may recognize a greater spectrum of antigenic determinants from RSV compared to their component homozygotes. Alternatively, heterozygotes may recognize the same antigenic determinants more efficiently (21, 23). Thus, the understanding of the mechanisms of genetic complementation may help in the development of genetically resistant breeds. The majority of the haplotypes in the SDL population were present in the heterozygous condition. The higher variability at the MHC proteins in the heterozygous condition may produce a survival advantage for this SDL stock in their native environment. Moreover, the results of the present study indicate that the breeding of chicks with haplotypes such as B^{2}, B^{21} and B^{22} may help generating RSV-A resistant flocks.

7. ACKNOWLEDGMENTS

The facilities provided by Director CARI, Izatnagar and Director IVRI, Izatnagar for carrying

out the investigation are thankfully acknowledged. We thank Dr. Charles R. Ashby, Jr. (St. John's University, Queens, NY) for editorial assistance.

8. REFERENCES

1. Witter RL, Bacon LD, Hunt HD, Silva RE, Fadly AM: Avian leukosis virus subgroup J infection profiles in broiler breeder chickens: association with virus transmission to progeny. *Avian Dis*, 44:913-931 (2000)
DOI: 10.2307/1593066
PMid:11195648

2. van Regenmortel MH, Mayo MA, Fauquet CM, Maniloff J: Virus nomenclature: consensus versus chaos. *Arch Virol*, 145:2227-2232 (2000)
DOI: 10.1007/s007050070053
PMid:11087105

3. Gao Y, Yun B, Qin L, Pan W, Qu Y, Liu Z, Wang Y, Qi X, Gao H, Wang X: Molecular epidemiology of avian leukosis virus subgroup J in layer flocks in China. *J Clin Microbiol*, 50:953-960 (2012)
DOI: 10.1128/JCM.00956-12
DOI: 10.1128/JCM.06179-11
PMid:22205787 PMCid:PMC3295186

4. Zekarias B, Ter Huurne AA, Landman WJ, Rebel JM, Pol JM, Gruys E: Immunological basis of differences in disease resistance in the chicken. *Vet Res*, 33:109-125 (2002)
DOI: 10.1051/vetres:2002001
PMid:11944802

5. Praharaj NK, Gross WB, Dunnington EA, Siegel PB: Feeding regimen by sire family interactions on growth, immunocompetence, and disease resistance in chickens. *Poult Sci*, 75:821-827 (1996)
DOI: 10.3382/ps.0750821
PMid:8805197

6. Briles WE, Stone HA, Cole RK: Marek's disease: effects of B histocompatibility alloalleles in resistant and susceptible chicken lines. *Science*, 195:193-195 (1977)
DOI: 10.1126/science.831269
PMid:831269

7. Lamont SJ: The chicken major histocompatibility complex in disease resistance and poultry breeding. *J Dairy Sci*, 72:1328-1333 (1989)
DOI: 10.3168/jds.S0022-0302(89)79240-7

8. Yoo BH, Sheldon BL: Association of the major histocompatibility complex with avian leukosis virus infection in chickens. *Br Poult Sci*, 33:613-620 (1992)
DOI: 10.1080/00071669208417500
PMid:1322760

9. Bacon LD, Witter RL, Crittenden LB, Fadly A, Motta J: B-haplotype influence on Marek's disease, Rous sarcoma, and lymphoid leukosis virus-induced tumors in chickens. *Poult Sci*, 60:1132-1139 (1981)
DOI: 10.3382/ps.0601132
PMid:6267578

10. Taylor RL, Jr.: Major histocompatibility (B) complex control of responses against Rous sarcomas. *Poult Sci*, 83:638-649 (2004)
DOI: 10.1093/ps/83.4.638
PMid:15109061

11. Collins WM, Briles, W.E. and Zsigray, R.M. : The B locus (MHC) in the chicken: association with the fate of RSV-induced tumors. *Immunogenetics*, 5:333-343 (1977)
DOI: 10.1007/BF01570490

12. Fulton JE, Juul-Madsen HR, Ashwell CM, McCarron AM, Arthur JA, O'Sullivan NP, Taylor RL, Jr.: Molecular genotype identification of the Gallus gallus major histocompatibility complex. *Immunogenetics*, 58:407-421 (2006)
DOI: 10.1007/s00251-006-0119-0
PMid:16738938

13. McConnell SK, Dawson DA, Wardle A, Burke T: The isolation and mapping of 19 tetranucleotide microsatellite markers in the chicken. *Anim Genet*, 30:183-189 (1999)
DOI: 10.1046/j.1365-2052.1999.00454.x
PMid:10442979

14. Livant EJ, Brigati JR, Ewald SJ: Diversity and locus specificity of chicken MHC B class I sequences. *Anim Genet*, 35:18-27 (2004)
DOI: 10.1111/j.1365-2052.2003.01078.x
PMid:14731225

15. Suzuki K, Kobayashi E, Yamashita H, Uenishi H, Churkina I, Plastow G, Hamasima N, Mitsuhashi T: Structural analysis of MHC alleles in an RSV tumour regression chicken using a BAC library. *Anim Genet*, 43:348-351 (2012)
DOI: 10.1111/j.1365-2052.2011.02247.x
PMid:22486511

16. Groupé, V., Dunkel, V. C., and Manake, R. A. Improved pock counting method for the titration of rous sarcoma virus in embryonated eggs. *J Bacteriol.* Sep; 74(3): 409–410 (1957).

17. Collins WM, Zervas, N.P., Urban, W.E. Jr., Briles, W.E., Aeed, P.A.: Response of B complex haplotypes B22, B24, and B26 to Rous sarcomas. *Poultry Science*, 64:2017-2019 (1985)
DOI: 10.3382/ps.0642017
PMid:2999742

18. Schou TW, Permin A, Juul-Madsen HR, Sorensen P, Labouriau R, Nguyen TL, Fink M, Pham SL: Gastrointestinal helminths in indigenous and exotic chickens in Vietnam: association of the intensity of infection with the Major Histocompatibility Complex. *Parasitology*, 134:561-573 (2007)
DOI: 10.1017/S0031182006002046
PMid:17166322

19. Lima-Rosa CA, Canal CW, Streck AF, Freitas LB, Delgado-Canedo A, Bonatto SL, Salzano FM: B-F DNA sequence variability in Brazilian (blue-egg Caipira) chickens. *Anim Genet*, 35:278-284 (2004)
DOI: 10.1111/j.1365-2052.2004.01160.x
PMid:15265066

20. Lwelamira J, Kifaro GC, Gwakisa PS: Genetic parameters for body weights, egg traits and antibody response against Newcastle Disease Virus (NDV) vaccine among two Tanzania chicken ecotypes. *Trop Anim Health Prod*, 41:51-59 (2009)
DOI: 10.1007/s11250-008-9153-2
PMid:19052902

21. Senseney HL, Briles WE, Abplanalp H, Taylor RL, Jr.: Allelic complementation between MHC haplotypes B (Q) and B17 increases regression of Rous sarcomas. *Poult Sci*, 79:1736-1740 (2000)
DOI: 10.1093/ps/79.12.1736
PMid:11194035

22. Brown DW, Collins WM, Ward PH, Briles WE: Complementation of major histocompatibility haplotypes in regression of Rous sarcoma virus-induced tumors in noninbred chickens. *Poult Sci*, 61:409-413 (1982)
DOI: 10.3382/ps.0610409
PMid:6283511

23. Collins WM, Briles, W.E., Corbett, A.C., Clark, K.K., Zsigray, R.M. and Dunlop, W.R.: B locus (MHC) effect upon regression of RSV-induced tumors in noninbred chickens. *Immunogenetics*, 9:97–100 (1979)
DOI: 10.1007/BF01570396

24. Lukacs NW, Briles WE, Briles RW, Taylor RL, Jr.: Response of major histocompatibility (B) complex haplotypes B22, B26, and B30 to Rous sarcomas. *Poult Sci*, 68:233-237 (1989)
DOI: 10.3382/ps.0680233
PMid:2539589

25. Taylor RL, Jr., Clare RA, Ward PH, Briles RW, Briles WE: Anti-Rous sarcoma response of major histocompatibility (B) complex haplotypes B23, B24 and B30. *Anim Genet*, 19:277-284 (1988)
DOI: 10.1111/j.1365-2052.1988.tb00816.x
PMid:2849889

26. Halpern MS, Ewert DL, Flores LJ, Fujita DJ, Aldrich CE, Mason WS: Sarcoma growth in 15I5 x 7 (2) chickens infected with avian sarcoma viruses of subgroup B or G. *Virology*, 134:472-476 (1984)
DOI: 10.1016/0042-6822(84)90315-5

27. Collins WM, Dunlop, W.R., Zsigray, R.M., Briles, R.W., Fite, R.W. : Metastasis of Rous sarcoma tumors in chickens is influenced by the major histocompatibility (B) complex and sex. *Poult Sciences*, 65:1642-1648 (1986)
DOI: 10.3382/ps.0651642
PMid:3022275

28. Sastri GA, Rao SP, Narayana JV, Rao PR, Christopher J: Histiocytic-Sarcomas in Domestic Fowls. (a Report of Five Cases) *Indian J Pathol Bacteriol*, 52:277-281 (1964)
PMid:14247636

29. Arshad SS, Howes K, Barron GS, Smith LM, Russell PH, Payne LN: Tissue tropism of the HPRS-103 strain of J subgroup avian leukosis virus and of a derivative acutely transforming virus. *Vet Pathol*, 34:127-137 (1997)
DOI: 10.1177/030098589703400205
PMid:9066079

Key Words: Major histocompatibility complex, Rous sarcoma virus–A, synthetic dam line, tumors, susceptibility, resistance, metastasis

Send correspondence to: Vishwa M. Khare, Cell and Developmental Biology, School of Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA, Tel: 215-971-6813, Fax: 215-898-987, E-mail: khare_vm@yahoo.com